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Alternative splicing and extra-telomeric role of telomerase reverse transcriptase isoforms in human embryonic stem cells

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Graduate Program in Physiology and Pharmacology
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Abstract

Human telomerase, a ribonucleoprotein complex consisting of a catalytic subunit (hTERT) and a RNA template component (TERC), prevents telomere attrition in human embryonic stem cells (hESCs) providing these cells with prolonged replicative capacity. Interestingly, hTERT has several alternatively spliced isoforms, most of which are catalytically inactive. One alternative splicing event involving a complete loss of exons 7 and 8 results in a catalytically inactive isoform called β -hTERT. The β -hTERT is highly expressed in certain cancer types and hESCs. Full-length hTERT (FL-hTERT) is known to translocate from nuclei to mitochondria when cells are exposed to either chronic (oxygen tension) or acute (hydrogen peroxide) oxidative stress. In this study, I examined the alternative splicing of endogenous hTERT transcript in hESC, intra-cellular localization of FL-hTERT and β -hTERT in hESCs, and the effect of overexpressing recombinant hTERT on hESC mitochondria. Human ESCs (HES2 line) were cultured under 2% oxygen and 3xFLAG tagged FL-hTERT and β -hTERT were induced by doxycycline prior to 100 μ M H₂O₂ treatment. My results demonstrate that overexpression of transgenic FL-hTERT resulted in a significant increase in endogenous β -hTERT transcript level. Additionally, doxycycline and H₂O₂ altered the splicing pattern of hTERT in favor of β -hTERT. I demonstrate that not only the full-length- but also β -hTERT can bind TERC, and that β -hTERT overexpression inhibits telomerase activity *in vitro*. However, I failed to observe the mitochondrial localization of recombinant FL-hTERT or β -hTERT. Lastly, my results demonstrate the pro-apoptotic role of β -hTERT and the pro-survival effect of FL-hTERT under H₂O₂-induced oxidative stress.

Key Words: Telomerase catalytic subunit, alternative splicing, oxidative stress, doxycycline

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List of Abbreviations

ALT	Alternative lengthening of telomere
APC	Adenomatosis polyposis coli
BIO	6-bromindirubin-3-oxime
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
Abl	Abelson murine leukemia viral oncogene homolog 1
CHIP	C terminus of HSC70-Interacting Protein
CRM1	Chromosomal maintenance 1
Ct	Cycle threshold
DHS	DNase I hypersensitive site
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-stranded break
ESC	Embryonic stem cell
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
ETC	Electron transport chain
FBS	Fetal bovine serum
FL	Full length
GSK3β	Glycogen synthase kinase 3 beta
hESC	Human embryonic stem cell

HIF1A	Hypoxia-inducible factor 1 alpha
HIF2A	Hypoxia-inducible factor 2 alpha
HK2	Hexokinase 2
hnRNP	Heterogeneous ribonucleoprotein
hPSC	Human pluripotent stem cell
HSP90	Heat shock protein 90
iPSC	Induced pluripotent stem cell
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
LRP	Lipoprotein receptor-related protein
MEF	Mouse embryonic fibroblast
MFN1/2	Mitofusin 1/2
MKRN1	Makorin ring finger protein 1
MMP	Mitochondria membrane potential
mtDNA	Mitochondrial DNA
mTHF	L-methyl-tetrahydrofolate
mTOR	Mechanistic target of rapamycin
NLS	Nuclear localization signal
NMD	Nonsense mediated decay
NP-40	Nonidet-P40
NS	Nucleostemin
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
Plk1	Polo-like kinase 1
PP2A	Protein phosphatase 2A
PSC	Pluripotent stem cell
PTB	Polypyrimidine tract binding protein
PTDC	Pyrrolidinethiocarbamate
RdRP	RNA-dependant RNA Polymerase
RISC	RNA-induced silencing complex
RMRP	RNA component of the mitochondrial RNA-processing endoribonuclease
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RT	Reverse transcriptase
S1P	Sphingosine-1-phosphate
siRNA	Small-interfering RNAs
snRNP	Small nucleolar ribonucleoprotein
SSEA	Stage-specific embryonic antigen
TBE	Tris/Borate/EDTA
TCA	Tricarboxylic acid
TCF/LEF	T-cell factor/lymphoid enhancer factor

TDH	Threonine dehydrogenase
TEN	Telomerase essential N-terminal
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
THF	Tetrahydrofolate
TNF	Tumor necrosis factor
UCP2	Uncoupling protein 2

Chapter 1: Introduction and Literature Review

1.1 Human Embryonic Stem Cells

In 1998, Jamie Thomson isolated human embryonic stem cells (hESCs) from the inner cell mass of a day 5-6 blastocyst [1]. Two defining characteristics of hESCs that define their therapeutic potential are their indefinite self-renewal capability and their ability to differentiate into cell types that comprise all three germ layers (endoderm, mesoderm, ectoderm) of a developing embryo including the germ cell lineage. Morphologically, hESCs grow in tight, spherical colonies when cultured on plastic dishes. Their cell size is small with very large nuclei compared to a small cytoplasmic space. hESCs are positive for various pluripotency markers such as alkaline phosphatase activity, pluripotency transcription factors (such as OCT4, NANOG, SOX2), glycolipids (stage specific embryonic antigens SSEA-3 and SSEA-4), and keratan sulfate antigens (Tra-1-60 and Tra-1-81) [2]. For their pluripotent nature and its stark resemblance to other immortal cell types, hESCs stand in the centre of research for human development, aging, cancer, and regenerative medicine. To address the ethical and technical difficulties associated with the derivation of hESCs, it was demonstrated that pluripotency could be induced directly from differentiated somatic cells of mice [3] and humans [4,5]. Despite many studies confirming the differentiation competency of these so called induced-pluripotent stem cells (iPSCs), iPSCs vary significantly in their self-renewal capacity due, in part, to their heterogeneity in telomerase activity levels and/or telomere lengths [6,7]. Human ESCs, like other PSCs, ubiquitously express high levels of telomerase activity [8]. In support of this, hESCs have a unusually long S phase of the cell cycle where telomere elongation by telomerase takes place [9] and have distinctive cell properties such as heavy reliance on glycolytic metabolism [10-13], and elevated DNA-repair/defenses [14-16].

1.1.1 Signaling pathways in hESCs

Typical PSC media for feeder-free growth conditions includes factors such as insulin-like growth factor (IGF) a strong stimulant for the canonical phosphatidylinositol-3 kinase (PI3K) signaling pathway [17,18]. PI3K/AKT signaling is crucial for maintenance of hESC pluripotency [19, 20]. PI3K/AKT is a signaling pathway best known for its regulatory role in cell cycle regulation. Its function is especially important in actively proliferating cell types such as cancer cells and hESCs. A microarray experiment by Armstrong *et al.* revealed a highly active PI3K/AKT signal-transduction in undifferentiated ESCs [18]. Inhibition of PI3K/AKT in hESCs resulted in a significant loss of core pluripotency marker (OCT4, NANOG, SOX2) expression, confirming the requirement of PI3K signaling in the maintenance of hESC pluripotency. When activated, AKT1 initiates a signaling cascade by activating mTOR and by inhibiting GSK3[19]. Inhibition of PI3K/AKT signaling by small molecules such as LY294002 or by removal of PI3K activators leads to an eventual loss of pluripotency in hPSCs [19, 20, 23]. The mechanisms of how PI3K controls the pluripotent state in hPSCs are unclear. It is speculated that PI3K/AKT regulates protein synthesis through mTOR, thereby regulating the protein synthesis of core factors of the pluripotency network [21]. Inhibition of mTOR by rapamycin results in similar effects to that of AKT inhibition, triggering spontaneous hPSC differentiation [19, 24]. GSK3 inhibition by AKT signaling may also contribute to PSC maintenance through Wnt/ β -catenin-mediated expression of c-myc, a key regulator of PSC maintenance [25, 26]. This mechanism could also apply in hPSCs, but has not been evaluated fully.

The role of canonical Wnt/ β -catenin pathway in human ESCs is a controversial one. At first, the activation of Wnt/ β -catenin pathway via either Wnt3A (ligand of Wnt-receptor) or GSK3 inhibitor 6-bromoindirubin-3'-oxime (BIO) was shown to have a significant role in

the self-renewal of hESCs (H1 and H9 cell line) under feeder-free conditions [27-29]. In presence of BIO, Wnt-signaling sustained the expression of Oct-3/4, Rex1, and Nanog. The effect was reversed when BIO was withdrawn from the culture, resulting in spontaneous multi-differentiation of hESCs. However, the consequences of blocking GSK3 are not limited to Wnt/ β -catenin pathway, and can affect many other pathways through mTOR stabilization/cross-talk between signaling pathways. For instance, inhibition of GSK3 via BIO results in increased phosphorylated Smad2/3 in hESCs, a downstream Nodal signaling pathway characteristic of the undifferentiated state of hESCs [26]. In support of this view, contradicting results emerged afterwards, demonstrating that Wnt/ β -catenin activation by Wnt3A/BIO does not support the self-renewal of hPSCs in long term cultures [30-32]. Moreover, their functional reporter assay showed that β -catenin-mediated transcription activity was nearly absent in undifferentiated hESCs (H1 and H9 cell lines). Further studies with a chimeric (estrogen-receptor tagged) β -catenin model showed that 4-hydroxytamoxifen-induced activation of Wnt/ β -catenin signaling resulted in the differentiation of hESCs into primitive streak and mesoderm progenitors [33, 34]. Inhibition of Wnt/ β -catenin signaling by blocking the secretion of Wnt [31] or by stabilizing β -catenin destruction complex [28] augments the self-renewal and long term propagation of human ESCs. It was additionally shown that Wnt-signaling promotes cell proliferation of hESC with little effect on apoptosis. This agrees well with the known gene targets upregulated by Wnt-signaling pathway such as cyclin D1 [32, 33], and c-Myc [34]. In short, while Wnt-pathway's pro-proliferative effect may be important in self-renewal of hESC, its importance in the maintenance of pluripotent state is unclear.

1.1.2 The role of oxygen tension in embryonic stem cells.

The natural niche of early embryonic cells is enriched with essential growth factors and the proper microenvironment for the maintenance of self-renewal and pluripotency, and this often includes low oxygen tensions [36, 37]. Early embryonic cells grow under low oxygen availability starting from fertilization and continuing through implantation and fetal development. During implantation, a hypoxic environment is created due to the absence of oxygen provided from maternal circulation [37]. The uterine surface typically has oxygen concentrations of 2% in early pregnancy. The oxygen tension rises to approximately 8% once the maternal vasculature is established [38]. Oxygen acts as the final electron carrier in the ETC of OXPHOS-dependent energy metabolism in mammalian cells [39]. During early embryo development, cells adapt to the low oxygen environment by switching between OXPHOS-dependent and glycolysis-dependent energy production [40].

hESCs derived from the early embryo grow relatively well under ambient oxygen tension (~21%) without apparent negative consequences. However, closer examination revealed the effect of oxygen tension on altered hESC morphology [42-44], pluripotency and differentiation marker expression [44, 45], cloning efficiency [43], chromosomal stability [44] and differentiation capacity [42, 43]. In the majority of the studies, low oxygen tension (2–5%) improved human ES cell pluripotency and maintenance [44– 47]. In addition, low oxygen condition (5%) was shown to improve the efficiency of iPS generation from human dermal fibroblast cells [48], further supporting the notion that stemness of hESC is augmented by hypoxia. Notably, conflicting results do exist to suggest that oxygen tension has minimal to no effect on the stemness of hESCs [43, 48].

In murine ESCs, hypoxia-inducible-factor1 alpha (HIF1A) plays a central role in regulating the cellular response to oxygen availability, mediating the expression of many oxygen-related genes [50]. While HIF1A controls a broader spectrum of oxygen-dependent genes, HIF2A is a direct upstream regulator of POU5F1 (OCT4) in mouse ES cells [51]. In hESCs, HIF1A is only transiently expressed for approximately 48 hours following hypoxic exposure [52], suggesting a long-term hypoxic regulation by a factor other than HIF1A. Indeed, HIF2A was described to be responsible for the regulation of energy metabolism and self-renewal in hESCs grown under 5% oxygen tensions [53].

1.1.3 Energy Metabolism of Pluripotent Stem Cells

Prior to vasculature formation, early-stage embryonic cells grow in a hypoxic environment (1-5%) [38, 53]. PSCs such as hESCs and mouse epiblast-derived stem cells (mEpiSC) exhibit low-level cellular respiration and down-regulation of electron transport chain (ETC) activity, while enzymes involved in glycolytic pathways are up-regulated [11]. This reliance on glycolytic metabolism is partly explained by the hypoxic micro-environmental condition of pre-implantation embryos [55]. Consequently, ATP synthesis in PSCs is primarily produced via glycolysis and pyruvate is converted into lactate instead of being fed into the Krebs cycle [13, 55]. hESCs' reliance on glycolysis is achieved by up-regulation of Glut1/3 [57], hexokinase2 (HK2) [55], phosphofructokinase (PFK) [58], and inactivation of mitochondrial ETC enzymes such as pyruvate dehydrogenase (PDH) [55]. Moreover, hESCs up-regulate UCP2 uncoupler protein, which in turn suppresses oxidative phosphorylation (OXPHOS) by actively shunting out C4 metabolites from mitochondria matrices [59]. In agreement with the suppressed mitochondrial glucose oxidation, mitochondria of hESC are structurally and functionally immature with underdeveloped cristae [55]. Conversely, differentiation of hESC is accompanied by rapid maturation of

mitochondria and increased metabolism via OXPHOS [60]. Glycolytic energy metabolism of hESCs serves to protect the cells from DNA damage by minimizing reactive oxygen species (ROS) generated through OXPHOS [55]. Although oxygen consumption is decoupled from ATP generation in ESCs, some level of oxygen consumption appears to be important, possibly to maintain functional tricarboxylic acid (TCA) cycle for lipid and protein synthesis by NADH oxidation to NAD⁺. Interestingly, PSCs do not experience a metabolic shift from glycolysis to OXPHOS in presence of sufficient oxygen, which closely resembles Warburg metabolism in cancer cells [55]. In the context of cancer cells, the aerobic glycolysis provides an anabolic advantage to support rapid cell growth. It is speculated that glycolysis endows a similar advantage in PSCs, whose key defining characteristic is high proliferative capacity. For instance, mouse ESCs show increased activity in pentose phosphate pathway and use it to generate carbon intermediates, which allows rapid nucleic acid synthesis [61]. Moreover, 3-phosphoglycerate can be converted into 3-phosphoserine and eventually into serine/glycine to be used for nucleotide biosynthesis and mono-carbon metabolism. One of the defining hallmarks of the Warburg phenomenon is rapid glutamine consumption that is used as an alternative carbon source for TCA cycle. Glutamine is the most abundant free amino acid in human plasma [62]. Glutamine is first converted into glutamate and subsequently to a key carbon component of TCA cycle alpha-ketoglutarate. The carbon intermediates generated through TCA minimally contribute to OXPHOS-dependent ATP synthesis. Instead, they are used for anabolic processes such as lipid (from citric acid pathway) and amino acid synthesis (alpha-ketoglutarate, oxaloacetate). In addition to methionine, threonine is another amino acid critical for mESC metabolism and proliferation. Threonine contributes to the pool of acetyl-CoA and glycine through L-threonine dehydrogenase (TDH) in mitochondria. Glycine then contributes directly to nucleotide biosynthesis or indirectly to S-adenosyl-methionine

(SAM) production by modulating tetrahydrofolate's (THF) conversion to L-methyl-tetrahydrofolate (mTHF). Threonine deprivation in mESCs leads to the reduction in the trimethylation of histone 3 lysine 4, increasing the propensity to differentiate [63].

The importance of mitochondria is also seen in reprogramming of somatic cells to induced pluripotent stem cells (iPSCs). During conversion of somatic cells to a pluripotent state, depleting mitofusin proteins (MFN1/2) promotes the reprogramming efficiency by increased mitochondria fission [64]. Similarly, the reprogramming efficiency of somatic cells into iPSCs is enhanced three- to four-fold when glycolysis is promoted, and is reduced when glycolysis is inhibited [48]. Several pieces of evidence suggest that mitochondrial maturation and subsequent rise in intra-cellular level of reactive oxygen species (ROS) are required signaling elements in regulating both stem cell function and differentiation potential [54, 63–65]. Conversely, use of potent antioxidant vitamin C improves somatic cell reprogramming efficiency by reducing intra-cellular ROS and by modifying the epigenetic landscape [68]. The significance of mitochondrial function and nuclear-mitochondrial cross-talk in stem cell biology remains yet poorly understood. The functional significance of mitochondria-dependent metabolites in the self-renewal, pluripotency, and induction of PSCs calls for a closer investigation.

1.1.4 Telomerase and Telomere in Human Embryonic Stem Cells

Telomeres are linear DNA structures at the end of chromosomes. They are non-coding DNA buffer regions that shorten with each DNA replication event due to the “end-replication problem” of eukaryotic DNA polymerase. For actively proliferating cells such as hESCs, maintaining the telomere homeostasis is critical for the function and longevity of the cells. A few mechanisms exist to maintain telomere lengths. In mice, both the telomerase and

alternative lengthening of telomere (ALT) pathway are utilized to maintain telomere lengths. In human cells, ALT is utilized to a lesser extent and telomerase activity is the primary means for maintaining telomere homeostasis. A ribonucleoprotein called telomerase is highly upregulated in hESCs for this reason, and the stable telomerase activity and long telomere lengths are characteristic of hESCs. Telomere lengthening via telomerase is activated as early as the blastocyst stage of embryo development in mice and high telomerase expression becomes absent in most adult somatic cells with exception of the germ line [69] and activated lymphocytes [65-69]. Of note, several stem cell types, their proliferative capacity notwithstanding, exhibit low to moderate telomerase activity and experience telomeric shortening. To date, most telomerase/telomere studies have been conducted with mouse embryonic stem cells (mESCs). In mESCs, the loss of telomerase activity results in telomere shortening, genomic instability, and reduced growth rates. Moreover, PSCs with long telomere lengths exhibit higher chimera success rate in tetraploid complementation assays than those with short telomeres [72]. These results suggests that telomere maintenance by telomerase is crucial for the proper functioning of pluripotent stem cells.

1.2 Human Telomerase Catalytic Subunit hTERT

1.2.1 Canonical Function of hTERT

The telomerase ribonucleoprotein (RNP) complex was first discovered in *Tetrahymena* by Elizabeth Blackburn and Carol W. Greider [73]. The human homologue of telomerase RNP was soon characterized [74], and was found to consist of catalytic protein subunit hTERT (127kDa) and RNA component called hTERC [75]. The active form of the telomerase complex requires dimerization of these components, and lack of either of these

components or the failure to dimerize result in loss of telomerase activity [76]. hTERC is a non-coding RNA that acts as a template for telomere sequence elongation. Using hTERC, hTERT adds multiple hexameric DNA repeats (TTAGGG) to the telomere ends. This canonical function of telomerase is strictly regulated at multiple levels. Transcriptionally, the pre-messenger RNA of hTERT gets spliced to generate various alternative transcripts (23 different alternatively spliced transcripts to date) with disrupted catalytic domain, ultimately resulting in a decreased pool of mRNA for catalytically active hTERT [75, 76]. Several studies have observed that the splicing pattern changes in a cell type-, and developmental stage-dependent manner, suggesting that alternative splicing is likely a tightly controlled regulatory mechanism for telomerase activity [77– 80]. However, the means or the effect of alternative splicing of hTERT on telomerase activity remains largely unknown (*see* section 1.2.2). Telomerase activity is also regulated post-translationally through phosphorylation and ubiquitination (*see* section 1.2.4). hTERT has a relatively short half-life of approximately 24 hours due to its fast-turnover rate through ubiquitination [83]. Interestingly, the RNA component hTERC is rarely the limiting factor for telomerase activity [73, 82]. Unlike hTERT, hTERC is ubiquitously expressed in most cell types, which appears to be independent of TERT levels [75]. However, in a pathological setting such as in the premature aging syndrome dyskeratosis congenita where TERC could be mutated or lacking, telomerase activity is inhibited [83, 84].

1.2.2 Alternative splicing of hTERT

Ninety five percent of protein coding genes are affected by alternative splicing in multicellular eukaryotes, generating >100,000 proteins from 20,000 protein-coding

sequences [87]. The canonical mode of RNA splicing entails joining of exonic sequences via the removal of the noncoding intron sequences. Splicing events occur on the nascent pre-mRNA, simultaneously with transcription [86, 87]. The event is orchestrated by a large and dynamic ribonucleoprotein called the spliceosome. A spliceosome is composed of snRNP core components (U1, U2, U4, U5, and U6) and other accessory proteins (~300 proteins) [90]. An exon may be constitutively included in the final transcript (constitutive splicing) or be conditionally included (alternative splicing), leading to alternative transcripts. Various local *cis*-regulatory sequences such as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs) regulate alternative splicing with the help of *trans*-acting elements such as snRNPs (activators), hnRNPs (repressors), and polypyrimidine tract binding protein (PTB)[89-91]. A clear understanding of splicing mechanisms is hampered by the vast number of splicing factors (>500), and by involvement of other cellular processes that affect spliceosome assembly. The selection of *cis*-elements is influenced by the molecular kinetics of transcription, 5'-end capping, and 3'-end polyadenylation [92, 93]. The complexity of alternative splicing is compounded by the competition of various transcripts for the splicing machinery [95].

The human TERT (hTERT) gene contains 15 introns and 16 exons and its transcript is alternatively spliced to produce various insertion and deletion isoforms [96]. Multiple alternative splicing events can occur on a single transcript, greatly expanding the pool of possible hTERT transcript variants. To date, 22 hTERT transcript variants have been identified [97]. However, translational competency of the identified alternatively spliced transcripts remains largely unknown. Amongst these, only the full-length hTERT (*hereafter*

referred to as FL-hTERT) constitutively spliced transcript (transcript with sixteen uninterrupted exons) results in a TERT protein capable of reverse transcriptase activity [97, 98].

An hTERT isoform called the β -deletion hTERT (*hereafter referred to as β -hTERT*) results from a 182-nucleotide out-of-frame deletion and is amongst the best characterized. The deletion skips exons 7 and 8, and joins exon 6 to exon 9 (*see* Fig. 1.1). The frame shift introduces a premature termination codon (PTC) and results in truncation of the hTERT protein [99, 100]. Splicing of β -hTERT is enhanced by serine/arginine-rich splicing factor 11 (SRSF11), and repressed by hnRNPH2 and hnRNPL [102]. Another study conducted by Wong *et al.* identified three highly conserved (amongst primates) direct repeat sequences in introns 6 and 8 [103]. The repeats in intron 6 (called *block 6* and *direct repeat 6* by the author) promoted β -deletion while the repeat in intron 8 (called *direct repeat 8*) repressed it. Importantly, block 6 is necessary for β -deletion splicing. Despite the PTC, it was reported that β -deletion transcripts escape non-sense mediated decay (NMD) and are translated into proteins [102]. There is currently no report on β -hTERT's propensity to degrade through protein degradation pathway. The β -hTERT contains an incomplete reverse-transcriptase domain and therefore was not considered a direct contributor to telomeric lengthening. However, Listerman *et al.* reported that β -hTERT acts as dominant-negative regulator of telomerase activity by sequestering hTERC [102]. Interestingly, β -hTERT transcript persists during human fetal development even when FL-hTERT splicing diminishes in various tissues in a development-stage-specific manner during fetal gestation [104]. Accumulating evidence indicates that either an increase or decrease in FL-hTERT transcript levels (and subsequent telomerase activity) may come at the expense of β -hTERT transcript abundance, further increasing the likelihood of regulatory functions by β -hTERT [100, 104-106].

The α -deletion hTERT (hereby noted as α -hTERT) results from a partial in-frame deletion (36bp) of exon 6, leading to the partial loss of RT-motif A. This catalytically inactive form of hTERT is a dominant negative inhibitor of telomerase activity when overexpressed in telomerase positive immortal cells [98, 107]. Considering the relatively low transcript abundance of the α -hTERT [108, 110], the level of telomerase activity inhibition by α -hTERT may be insufficient for it to have significant impact on telomere maintenance of indefinitely dividing cells. Following the discovery of the α -hTERT, Hisatomi *et al.* discovered another catalytically inactive variant (γ -hTERT) with two missing RT-motifs [110]. Like the α -hTERT, the γ -hTERT was suggested as a negative regulator of telomerase activity. However, γ -hTERT transcript levels, relative to those of α -hTERT and β -hTERT, are extremely low or nearly undetectable in all tested cell types [111-112]. Deletions at α and β sites can occur simultaneously on a single transcript generating truncated and catalytically inactive α - β -hTERT [112]. The function, splicing mechanisms, and protein stability of this isoform yet remain to be elucidated.

Telomerase activity requires dimerization of two catalytically active forms of hTERT [76]. Therefore, all translated hTERT splice variants that contain an oligomerization domain can potentially suppress telomerase and/or extra-telomeric functions of FL-hTERT by forming heterodimeric complex with it (Figure 1.1)

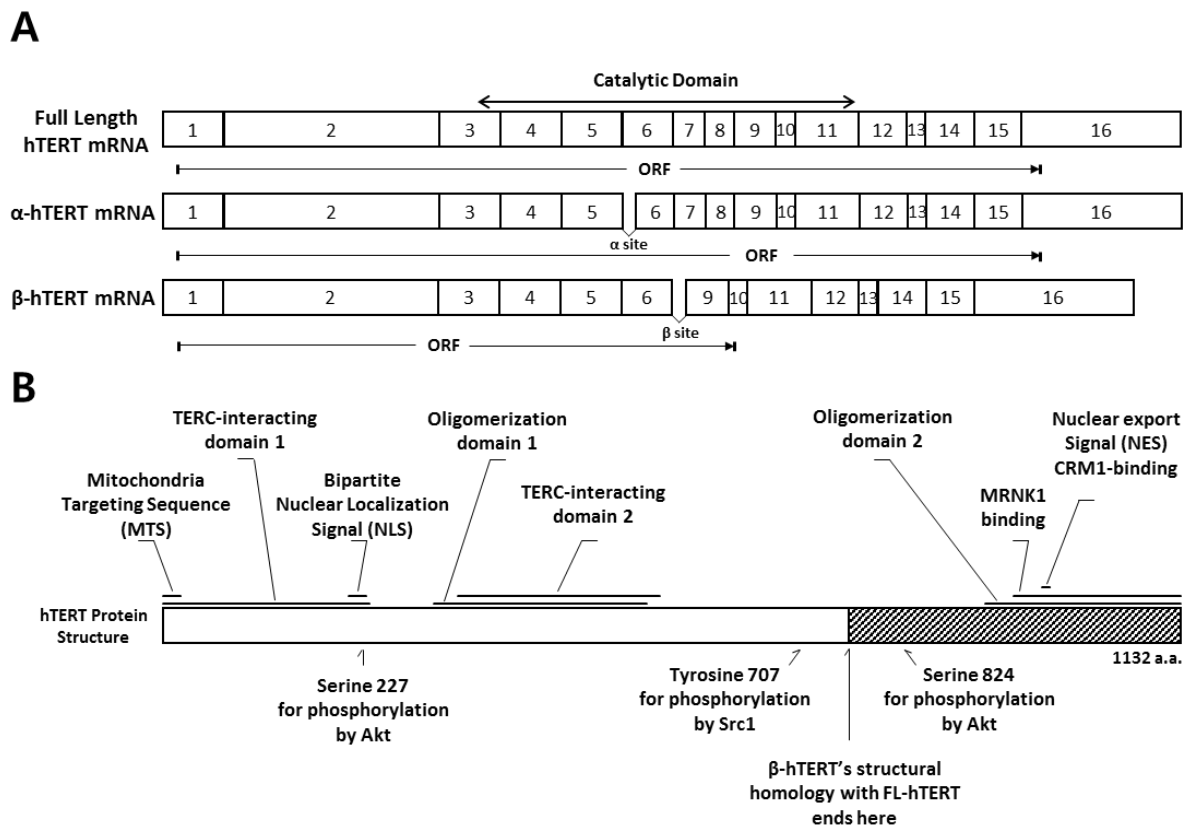


Figure 1.1: mRNA and protein structure of the major hTERT isoforms. A) Transcript structures of FL-hTERT, α -hTERT, and β -hTERT post-splicing. The deletion site and the open reading frame (ORF) of each isoform are indicated. B) Protein structure of hTERT. Regulatory domains/residues on the protein are annotated.

1.2.3 *Transcriptional Regulation of hTERT*

An intricate regulation of hTERT expression is orchestrated by several transcription factors and DNA/histone modifying complexes at the promoter region. HSP90 (whose involvement is important for telomerase DNA binding and association with hTERC [113], [123, 124] was found to interact with the hTERT promoter in immortalized and cancer cells, regulating hTERT transcription [116]. Inhibition of HSP90 causes hTERT transcription and telomerase activity to decrease, which occurs concurrently with the diminished interaction of HSP90 with the hTERT promoter [116]. It is postulated that HSP90 may associate with GC-box-binding transcription factors such as Sp1 [116]. GC-boxes are important regulatory regions of the hTERT promoter that lacks a TATA-box [126, 127]. The hTERT promoter contains a minimum of five GC-boxes, and mutations in these regions severely impair the transcriptional activity at the site [116]. Notably, Sp1's interaction with the hTERT promoter is not disrupted by inhibition of HSP90, but there has been a report on their cooperative coordination of other GC-box-containing gene expression [119], suggesting an auxiliary function of HSP90 in Sp1-driven hTERT transcription. The significance of Sp1 in hTERT regulation is a controversial one. As demonstrated by Cheng *et al.*, both Sp1 and its related transcription factor Sp3 are required at the hTERT promoter for its transcription [120]. This agrees with previous reports that Sp1-family proteins generally activate hTERT transcription. Conversely, the repressive role of Sp1/3 has also been reported in normal human somatic cells [121]. Notably, the epigenetic state of the hTERT promoter appears to have little effect on Sp1 binding. Conversely, binding of Sp1/3 does not affect H3K9 (histone 3 lysine 9) acetylation or trimethylation status. It is postulated that hTERT transcriptional activation/repression by Sp1/3 may be dependent on other cell-type/-state specific factors, and that the recruitment of the complex to the promoter is relatively insensitive to the

epigenetic state of the target site [121]. Nonetheless, the heterochromatin-like state of the hTERT gene must become more open landscape for transcription factors' access to the GC-boxes and the eventual promoter activity in telomerase positive cells [120].

E-box transcription factors, Myc family proteins, and USF1/2 are believed to be the key de-repressors of the hTERT promoter via the recruitment of histone-modifying complexes [120]. The E-box transcription factors were found to be clustered around the hTERT promoter, possibly to promote an open conformation of the site. However, such occupation by E-box transcription factors at the hTERT promoter is seen even in telomerase-negative cells. This implies an involvement of additional factors to initiate the remodeling of heterochromatins [120]. In the same study, it was demonstrated that the rate of hTERT transcription was directly proportional to the establishment of a nucleosome-free region (also known as a DNase I hypersensitive site (DHS; euchromatic) in the hTERT promoter of telomerase positive cells [120]. Chromatin remodeling events are not unique for GC-boxes as they can also take place at estrogen response elements (EREs) in the hTERT promoter of estrogen-receptor- α -positive cells [122]. In addition, retinoic acid hormone receptor family proteins can repress hTERT transcription by reducing H3K9 acetylation (change to heterochromatic state) and by promoting methylation at the promoter region (repressive marker) [132, 133]. Such hormonal control of hTERT transcription has significant impact on the up-regulation of hTERT by oncogenic transcription factors such as c-Myc and HIF1A. [134, 135], both of which are direct positive-regulators of hTERT transcription [136, 137]. While the transcriptional control by various transcription factors and chromatin remodeling proteins is an important step in hTERT regulation, mounting evidence indicates that post-translational modification mechanisms play critical roles in determining the end-function of the hTERT protein (discussed in the following section).

1.2.4 *Post-translational activation/repression of hTERT*

hTERT is primarily regulated at the transcriptional level [113, 114], but regulation by post-translational mechanisms, such as phosphorylation and ubiquitination, has been reported and thought to be important [113, 115]. New discoveries regarding the post-translational regulation of hTERT are continuously emerging.

Two known mechanisms of post-translational regulation of hTERT are by phosphorylation and ubiquitination (Figure 1.2). The best characterized degradation process of hTERT occurs through ubiquitination by Makorin Ring Finger Protein 1 (MKRN1, interacts with hTERT C-terminal residues 946–1132)[129], C terminus of HSC70-Interacting Protein (CHIP)[130], and HDM2 (E3 ligases)[131]. Ubiquitinated hTERT is subsequently targeted for destruction by proteasomes in the cytoplasm. The exact mechanisms of ubiquitination remain to be elucidated. The fact that the ubiquitination and degradation processes of hTERT take place in the cytoplasmic space makes hTERT's sub-cellular localization a critical factor in hTERT regulation. In line with this, one dominant negative form of hTERT was proposed to promote the degradation of FL-hTERT by exporting itself out of nuclei with the FL-hTERT (as heterodimeric complex), where hTERT becomes available for ubiquitination and for the subsequent proteolysis [132] in MCF-7 cells, ultimately reducing telomerase activity [133]. Sphingosine-1-phosphate (S1P) is another bioactive lipid mediator of hTERT degradation [134]. Allosteric binding of S1P to hTERT mimics a phosphorylated residue at aspartate 684, which in turn prevents the interaction of MKRN1 with hTERT (therefore stabilizes hTERT against proteolysis). It was also shown by the same group that a similar phosphomimetic substitution by S1P occurs at serine 921, protecting hTERT from degradation while keeping them in the nuclear region.

Activation of hTERT is primarily regulated by a series of phosphorylation events caused by various kinases and adaptor proteins. Phosphorylation of hTERT by protein kinase C (PKC) isoenzymes alpha, beta, delta, epsilon, and zeta phosphorylate hTERT, thereby enhancing telomerase activity [144, 145]. The mechanism of this remains unclear. hTERT contains a consensus bipartite nuclear localization signal (NLS) consisting of two clusters of basic amino acids ($^{222}RRR^{224}$ and $^{236}KRPRR^{240}$) [137]. Additionally, putative Akt-phosphorylation sites are located at $^{220}GARRRGGSAS^{229}$ and $^{817}AVRIRGKSYV^{826}$, and the phosphorylation of serine 227 (but not serine 824) is required for nuclear translocation of hTERT [138]. Chung et al. [137] predicted that the observed post-translational modification by Akt may result in a conformational change of hTERT, generating new interaction sites for binding partners like p65 of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) family proteins [139]. Similarly, Polo-like kinase 1 (Plk1) is believed to augment telomerase activity by promoting the nuclear retention of hTERT (thereby preventing the proteosomal degradation of hTERT in the cytoplasm) [140]. In support of this, overexpression of Plk1 resulted in increased hTERT protein levels without changing the steady-state transcript level of hTERT [140]. However, Plk1 does not phosphorylate hTERT, nor does it affect its interaction with hTERT [140]. Conversely, extra-nuclear localization of hTERT is regulated by the phosphorylation of Src tyrosine kinase at tyrosine 707 [141]. Src-mediated tyrosine 707 phosphorylation enables binding of Ran GTPase to hTERT, and the subsequent nuclear export via the chromosomal maintenance 1 (CRM1) export receptor [142]. 14-3-3 protein can bind hTERT and prevent its cytoplasmic localization by inhibiting CRM1-hTERT interaction [143]. Contrarily, dephosphorylation of hTERT by the phosphatase PP2A blocks the interaction between 14-3-3 and hTERT, thereby disrupting the nuclear localization and telomerase activity [144]. In the context of mitochondrial

localization, Src (mitochondrial)-mediated phosphorylation at Y707 was also shown to be responsible for the depletion of mitochondrial hTERT following H₂O₂-treatment [141]. In the same study, H₂O₂ treatment led to the reduced activity of mitochondria-resident Akt, suggesting Akt may regulate hTERT function not only in the nuclei, but also in the mitochondria [141]. Another tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (c-Abl) is a negative regulator of telomerase activity. c-Abl directly interacts with hTERT (at ³⁰⁸PSTSRPPRP³¹⁶) and induces tyrosine phosphorylation of hTERT in MCF-7 cells following ionizing radiation, reducing telomerase activity. However, the exact location of phosphorylated amino acid residue and the mechanism remain to be investigated [133].

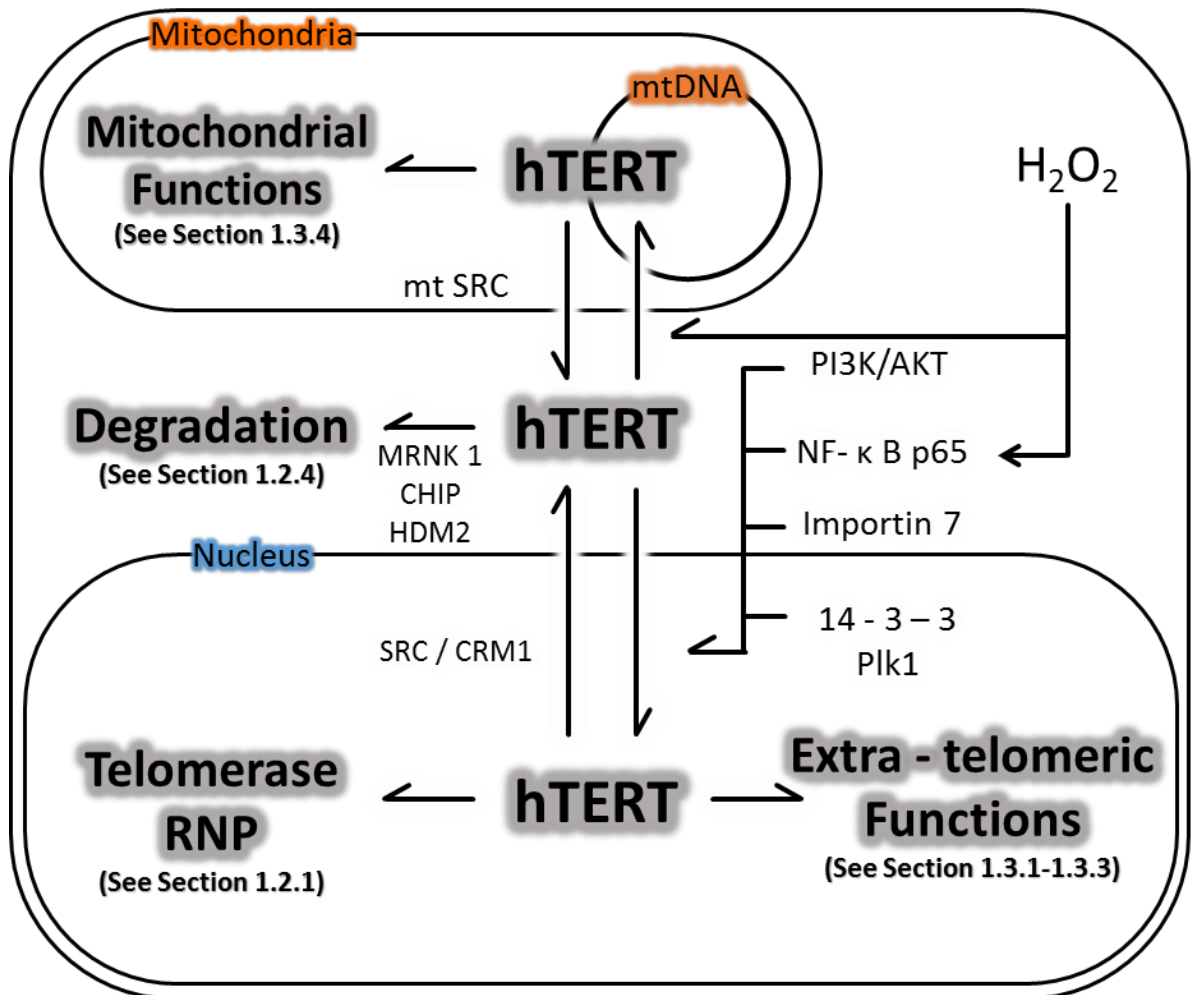


Figure 1.2: Post-translational modification and function of hTERT. Summary of signaling molecules/pathways that regulate the intra-cellular movement/stability of hTERT. hTERT is recruited to nuclear space for telomerase activity (section 1.2.1) and extra-telomeric functions (section 1.3.1-1.3.3). Conversely, hTERT is exported out of nucleus for ubiquitin-mediated degradation (section 1.2.4) or is targeted to mitochondria for mitochondrial functions (section 1.3.4). Factors/signaling-pathways that regulate hTERT translocation are indicated beside the arrows.

1.3 Non-canonical functions of Human Telomerase Catalytic Subunit hTERT

1.3.1 Non-Canonical Functions of hTERT in Wnt/ β -catenin pathway

hTERT participates directly with Wnt/ β -catenin to regulate downstream gene expression [145]. Wnt-signaling governs cell proliferation, and its involvement is critical for proper axis formation during embryo development. Three known modes of Wnt-signaling pathways are Wnt/calcium pathway, planar cell polarity pathway, and the canonical Wnt/ β -catenin pathway [145]. The canonical signaling cascade starts when extra-cellular signaling Wnt family proteins bind to the N-terminal extra-cellular cysteine-rich domain of Fizzled (Fz) family receptor and lipoprotein receptor-related protein (LRP)-5/6 [145]. The binding of Wnt to Fz and LRP-5/6 leads to the dephosphorylation of a negative regulator of Wnt-signaling called Axin. This subsequently causes the disruption of protein degradation machinery (consisting of adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 β (GSK3 β), which otherwise marks β -catenin for degradation by ubiquitination. The stabilized β -catenin eventually accumulates in nuclei to initiate the cellular response with the help of other transcription factors such as T-cell factor/lymphoid enhancing factor (TCF/LEF) and BRG1 [146]. It was demonstrated by Park *et al.* [147] that murine TERT (mTERT) modulates Wnt/ β -catenin by acting as a cofactor of transcription complexes that consists of TCF/LEF and BRG1 (SMARCA4). Another group also confirmed TERT's association with BRG1 (Brahma-related gene 1) in human cells [148]. However, a contradicting result presented by Elizabeth Blackburn's group [149] showed that hTERT did not associate with BRG1. The discrepancy may arise from cell type-, and species specific differences. Interestingly, β -catenin was found to be a positive and direct regulator of hTERT gene expression through the interaction with Klf4 (in mouse ES cells) [150], and TCF4 (in human cancer cell) [151] at the transcription start site, suggesting a feed-

forward regulation between Wnt-signaling and hTERT expression. BRG1, on the other hand, suppresses hTERT expression by forming a ternary complex with HDAC2, leading to deacetylation of H3K9ac and H4ac at the transcription start site of hTERT in human cancer cells [152].

1.3.2 Non-Canonical Functions of hTERT in NF- κ B pathway

Several groups have shown that TERT regulates NF- κ B-dependent gene transcription in the absence of any telomere-mediated effects [156, 166-168]. The NF- κ B signaling pathway is a master regulator of cell stress, inflammatory response, and developmental signaling [169- 171]. NF- κ B protein family proteins include p50, p52, RelA (p65), RelB, c-Rel. The canonical NF- κ B signaling involves dimerization of p65/p50 subunits (stabilized through the degradation of I-kappa-B-alpha (I κ B α)) to regulate the expression of target genes. NF- κ B is strongly activated in cancer cells, which led more researchers to investigate the potential regulatory cross-talk between hTERT and NF- κ B. The studies have revealed that NF- κ B positively regulates TERT by directly binding to its promoter [157]. In agreement, it was demonstrated that hTERT's nuclear translocation and telomerase activity/expression were enhanced by the binding of p65 in MM.1S cells [173, 174]. Additionally, Ghosh *et al.* recently discovered that hTERT directly regulates the expression of the NF- κ B-dependent genes by binding to p65, and localizing to a subset of NF- κ B sites, on promoters of IL-6, TNF- α and IL-8 [166, 167]. In the same study, results indicated that TERT is not merely a co-factor, but a requirement for optimal p65 binding to a few NF- κ B target genes when NF- κ B signaling was induced by TNF- α stimulation [153]. Ghosh *et al.* clarified this further by identifying telomere like sequences (TTGGG) within the promoter regions of IL6A, IL8A and TNF- α genes [153].

Not much is known about NF- κ B signaling with respect to the pluripotency and self-renewal of PSC. However, the gene expression/proteome profiling of undifferentiated and differentiated ESCs show that components of the NF- κ B signaling pathway are enriched in undifferentiated hESC, and downregulated during differentiation. Immunohistochemistry of RelA showed that it is only present in the nucleus of undifferentiated hESC, indicating active NF- κ B signaling. To test the potential importance of NF- κ B signaling in the maintenance of pluripotency, a specific NF- κ B inhibitor sodium pyrrolidinethiocarbamate (PTDC) was added to the cells. PTDC prevents RelA (p65) entry into the nucleus. Inhibition of NF- κ B signaling by PTDC caused massive cell differentiation within ESC colonies, as well as significant cell death, demonstrating that NF- κ B, or at least the nuclear translocation of hTERT mediated by NF- κ B molecule p65, is essential to maintain ESC pluripotency [18]. The downregulation of NF- κ B signaling was also observed in spontaneously differentiating human ESCs. In another study with three independent human iPSC lines, the augmented level of NF- κ B signaling was confirmed. Moreover, the knockdown of NF- κ B resulted in decreased Nanog and Oct-3/4 expression levels, suggesting an important role of NF- κ B in the maintenance of undifferentiated human PSCs [161]. In contrast, NF- κ B signaling is suppressed in undifferentiated mouse ESCs and activated in differentiating mouse ESCs [176, 177]. Torres *et al.* reported that a pluripotency transcription factor Nanog represses NF- κ B signaling to promote pluripotency. Taken together, the studies point to the importance of NF- κ B signaling and its cross-talk with hTERT in modulation of pluripotency in human PSCs.

1.3.3 Non-Canonical function of hTERT as an RNA-dependant RNA Polymerase (RdRP)

Several pieces of scientific evidence exist to show that TERT plays a direct role in the regulation of gene expression, including Wnt/ β -catenin and NF- κ B pathways just reviewed.

In more recent years, several reports show that TERT may regulate gene expression at the post-transcriptional level in an extra-telomeric fashion [157, 178, 179]. Maida *et al.* showed that hTERT can interact with non-TERC RNA species called RNA component of the mitochondrial RNA-processing endoribonuclease (RMRP) to form a ribonucleoprotein complex that has RNA-dependent RNA polymerase activity. The resulting reverse transcriptase TERT-RMRP complex generates double stranded RNA (dsRNA) using RMRP as a template RNA strand. The dsRNAs are subsequently processed by Dicer1 into 22-nucleotide small-interfering RNAs (siRNAs) that selectively cleave and silence themselves. RMRP is a 267 nucleotide-long non-coding RNA that is highly expressed in many human and murine tissues. Notably, RMRP expression is activated by Wnt/ β -catenin and YAP proteins in human carcinoma cells [166], suggesting TERT-RMRP complex may be a part of much broader signaling pathway. The canonical role of RMRP is implicated in mitochondrial DNA replication where it initiates the replication process by cleaving another mitochondrial RNA (inhibits progression of mitochondrial DNA replication) bound at the priming site [167]. Mutations in the RMRP gene are associated with cartilage hair hypoplasia (CHH) [182, 183]. Homozygous deletion of RMRP in mouse results in embryonic lethality, indicating RMRP is essential for early mouse embryonic development [165]. Sharma *et al.* (a team led by a pioneering scientist to show mitochondrial effects of hTERT) suggested the mitochondrial hTERT binds different mitochondrial RNAs and carries out its tentative mitochondrial functions (*see* section 1.2.4) in TERC-independent manner [170]. The study raised possibilities that there may be more RNA binding partners other than RMRP to regulate gene expression in the mitochondria.

Outside of mitochondrial context, hTERT was shown to regulate heterochromatin assembly at centromeres and transposon elements on chromosomes [148]. hTERT associates

with BRG1 and nucleostemin (NS) to form a TBN (TERT, BRG1, and Nucleostemin) complex with RdRP activity. The complex produces dsRNAs homologous to centromeric alpha-satellite (alphoid) repeat elements. The dsRNA is then processed into siRNAs that target the centromeric alphoid regions to promote the recruitment of heterochromatin assembly complexes to the site. In a different study by the same group, it was shown that siRNA generated via RMRP-hTERT RdRP and RNA-induced silencing complex (RISC) negatively regulated PHYHIPL transcript level whose cellular functions are yet unclear [171]. Even though there has been no report to indicate the functional significance of hTERT as TERC-independent RdRP in hESCs, the mounting evidence of it in other cell types suggest hTERT may carry out important nuclear and mitochondrial roles in hESCs independently of TERC.

1.3.4 Mitochondrial Functions of hTERT

Santos *et al.* (2003) [172] have shown that ectopically expressed TERT exacerbates H₂O₂-mediated mitochondrial damage in human cells. They delineated this phenomenon by identifying the putative 20 amino acid-long mitochondrial localization signal at the very N-terminal end of hTERT. hTERT associates with mitochondrial membrane translocases TOM20/40 and TIM23 proteins, further suggesting the mitochondrial localization of hTERT [173]. Mitochondrial DNA has 22 sites with telomere-like sequences for hTERT binding, and hTERT's binding to two of these sites has been confirmed [173]. The bound regions of mitochondrial DNA each code for complex I NADH-Ubiquinone Oxidoreductase chain 1 and 2 subunits [173]. Ectopic expression hTERT up-regulates complex I activity [173]. Conversely, ectopic expression of catalytically inactive hTERT results in decreased complex I activity. Other known mitochondrial effects of overexpressed FL-hTERT include increased mitochondrial membrane potential, down-regulated UCP2 (uncoupler of mitochondria that is

responsible for repressing OXPHOS in PSCs), reduced mitochondrial superoxide, mitochondrial DNA (mtDNA) content, and mitochondria mass [174].

It was suggested that ectopically expressed hTERT lead to increased H₂O₂-mediated mtDNA damage through increased availability of metal ions [175]. However, other studies demonstrate that FL-hTERT protects mtDNA from H₂O₂-induced oxidative stress [142], [174]. In addition, FL-hTERT protects mtDNA from other genotoxins such as ethidium bromide and UV-light. In general, hTERT appears to augment mitochondrial function and maturation. A growing body of research suggests that TERT has important effects on mitochondrial function in a telomere-independent manner [187, 190-193]. However, the mechanism by which TERT protects mitochondrial function is not completely understood, and remains to be elucidated

1.4 Rationale

Mitochondria in undifferentiated hESCs are localized to the peri-nuclear space, and remain low in copy number and are punctuate in morphology [60]. In contrast, hESC differentiation is accompanied by the formation of a reticular network of maturing mitochondria with a simultaneous increase in OXPHOS and ROS production [66]. Taken together, mitochondrial function and biogenesis is key to understanding the regulation of pluripotency in undifferentiated hESCs and in early differentiation processes [178].

hTERT can interact with a wide range of biomolecules such as DNA [187, 195], transcription factors [156, 166], a helicase [180], and various species of RNA [157, 185, 196-

198]. Thus, hTERT has various functions in the nucleus and mitochondria. Interestingly, a number of these hTERT functions require catalytically activity of hTERT [181] and is often inhibited by catalytically inactive forms [98, 108, 187]. Many past studies [175–177, 187, 188] and our lab's previous work by Radan *et al.* [184] demonstrated that high oxygen tension and oxidative stress on cells induce the extra-nuclear/mitochondrial localization of hTERT. Radan *et al.* additionally showed that pharmacological inhibition of telomerase activity, but not TERC, resulted in spontaneous differentiation of hESC. Similarly, disrupting β -splicing by morpholino oligonucleotides in hESC resulted in loss of pluripotency, indicating the importance of hTERT and its non-catalytic variants in stem cell function [184]. Yang *et al.* [185] had also shown the importance of hTERT in hESC maintenance through knockdown studies. β -hTERT, like its full-length counterpart, has an intact MTS [172], RNA-interacting domains [199, 200], and oligomerization sequence [188], and retains some phosphorylation sites for nuclear import [201, 152] and export [142] (Figure 1.1). Listerman and colleagues recently demonstrated that β -hTERT is capable of mitochondrial localization in HeLa cells [102]. However, the cause and the pattern of β -hTERT localization is currently unknown. This mitochondrial localization may allow β -hTERT to alter the function of FL-hTERT in any subcellular compartment by sequestering key functional components such as specific RNA components or by disrupting the formation of a functional homodimer of catalytically active hTERT subunits.

Following the relatively recent discovery of hTERT localization to mitochondria, several studies indicated that mitochondrial FL-hTERT may function through modulation of mitochondria biogenesis, retrograde response to oxidative stress, and changes in divalent metal ion homeostasis [186-188]. It was demonstrated that FL-hTERT interacts with mtDNA, and enhances mitochondrial function [173]. Notably, overexpression of a reverse-

transcriptase incompetent hTERT mutant abrogated this effect [173]. Considering β -hTERT is a reverse-transcriptase incompetent form, there may exist an interaction between FL-hTERT and β -hTERT in mitochondria to fine-tune mitochondria function, possibly through hetero-dimerization. Collectively, targeting of β -TERT to mitochondria and its potential to modulate mitochondrial function and growth may imply biological significance in the regulation of pluripotency, self-renewal and differentiation potential of PSCs.

Hypothesis: Extra-telomeric hTERT β -isoform localizes to mitochondria under H_2O_2 -induced oxidative stress, modulating mitochondria function in hESCs.

Objectives:

1. To characterize FL-hTERT and β -hTERT expression and their role in modulating telomerase activity in hESCs.
2. To characterize the mitochondrial localization pattern of FL-hTERT and β -hTERT in hESCs under H_2O_2 -induced oxidative stress conditions.
3. To determine the effect of overexpressed FL-hTERT and β -hTERT on mitochondrial biogenesis and function in hESCs.

Chapter 2: Material and Methods

2.1 Cell Culture

2.1.1 Mouse Embryonic Fibroblasts Feeder Cells

Mouse embryonic fibroblasts (MEF) secrete several soluble growth factors to enhance the self-renewal and pluripotency of hESCs. For this reason, hESCs are often co-cultured with irradiated MEF-feeder cells. Four drug (neomycin, hygromycin, puromycin, and 6-thioguanine)-resistant MEF(4DR-MEF) cells were purchased for the future use in drug-selection of cloned transgenic hESC populations. 4DR-MEF was cultured in DMEM medium (ThermoFisher) containing 10% fetal bovine serum, 1X Non-Essential Amino Acid (NEAA; ThermoFisher) under 5% CO₂ at 37°C. A bulk batch of MEFs was γ -irradiated to halt the cell proliferation and was cryo-preserved in small aliquots. γ -irradiated 4DR MEFs (approximately 60,000 cells and 10⁶ for each well of 6-well plate and 10cm dish, respectively) were thawed and allowed to settle onto the culture dishes or plates for one day prior to being used as feeder-layer for hESC culture.

2.1.2 Human Embryonic Stem Cell Line HES2

The human ESC line HES2 was originally established in a fertility clinic in Singapore [190] and was purchased from ATCC for utilization in this study. For maintenance purposes, HES2 cells were cultured on γ -irradiated 4DR MEFs layer for a single passage (3-4 days to reach 70-80% confluency). hESC growth medium (83% KnockOut™ DMEM/F-12 (Gibco™), 15% KnockOut™ serum replacement (ThermoFisher), 1% GlutaMax™ (X100; Gibco™), 1% NEAA (X100; Gibco™), 0.1% DMSO (Sigma), and 8ng/ml bFGF

(ThermoFisher)) was refreshed daily. The stably growing HES2 cells were subsequently expanded onto a feeder-free basement membrane Geltrex™ (Life technologies) in 2% O₂ (37°C, in 5% CO₂) for experimental use. Fresh Essential 8™ Medium (ThermoFisher) was supplied daily for feeder free hESC culture. Cells were treated with 100µM H₂O₂ for up to 4 hours.

2.1.3 Establishment of monoclonal transgenic HES2 lines.

The lack of a reliable hTERT-specific antibody has been a challenge in studying FL-hTERT, β-hTERT, and other TERT isoforms. To address this problem, my lab established clonally selected transgenic hESC cell lines capable of doxycycline-inducible overexpression of 3xFLAG-tagged FL-hTERT and 3xFLAG-tagged β-hTERT (hereby called HES2 ‘FL’ and HES2 ‘B’ for 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT lines, respectively), enabling the use of commercially available anti-FLAG antibody to trace/isolate overexpressed FL-hTERT and β-hTERT transgenic proteins in hESCs (Fig. 2.1 A). Briefly, endogenous FL-hTERT and β-hTERT transcripts were reverse-transcribed and PCR-amplified into library DNA sequence using hTERT specific primers (appendix A1: Table 2.1). The library hTERT sequences were extended to include Gateway LR-Clonase (ThermoFisher) adaptor sequences attL1 (5’GTACAAAAAAGCAGGCT), attL2 (TGGGTCGAAAGAACATG3’), 5’Kozak sequence (GCCGCCACC), 3xFLAG sequence (GACTACAAGGACCACGACGGTGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGTGA), and β-GEO drug resistance gene (appendix A1: Table 2.1) by PCR. The clone sequence of FL- and β-hTERT was ligated into the open reading frame of Tet-inducible/transposable entry vector of PiggyBac (PB) Transposon System (donated by

Nagy Lab, Mt Sinai Hospital). The correct orientation and sequence of ligated inserts was validated by DNA sequencing (Robarts Genomic facility). The entry vector plasmid and two other PB plasmid constructs containing PiggyBac rtTA gene (transactivator of gene of interest (GOI) expression; genome-integrated), and PiggyBac transposase gene (facilitates integration of GOI and rtTA into the genome; short-lived without genomic integration) were introduced to HES2 genome via electroporation (170V, 1050 μ F). Successfully transformed HES2 cells were selected by blasticidin (10 μ g/ml; 1 day) and G418 (1mg/ml; 7 days). The surviving cell clusters were single-celled and clonally picked to establish the final transgenic HES2 clones.

2.1.4 Cell treatment scheme

Preliminary experiments were carried out to determine the optimal treatment time and dose of doxycycline for the induction of hTERT transgene expression in HES2 clones (appendix A3: Fig 3.1). Non-transgenic HES2 (hereby referred to as HES2) and HES2 B and HES2 FL clones were treated with doxycycline-containing (1 μ g/ml) Essential 8 media for 2 days. The culture medium was renewed daily. Based on studies that demonstrated extra-nuclear localization of hTERT following oxidative stress treatments (H₂O₂ and/or high oxygen tension)[144, 174, 185], H₂O₂ (100 μ M)-containing growth medium was directly added to the culture 2 hours prior to cell harvest. Cells were kept under 2% oxygen tension for optimal cell growth of hESC (*see* section 1.1.2).

2.2 PCR protocols

2.2.1 General Real-Time quantitative PCR protocol

A phenol-chloroform extraction protocol was performed for RNA isolations as outlined in the product instructions (TRIzol reagent; ThermoFisher). Isolated RNA was reverse transcribed to cDNA using SuperScript III (ThermoFisher). cDNA concentrations were estimated with a NanoDrop (ThermoFisher) spectrophotometer and equal amount of template was ensured for each set of PCR reactions. RT-qPCR reactions consisted of SensiFAST™ (Bioline) PCR mix, gene-specific primer sets, and cDNA template. DNA polymerase was heat-activated at 95°C for 2 minutes prior to PCR cycles. Temperature cycling conditions were 95 °C for 10 seconds (denaturation), pre-determined temperature (primer specific; appendix A1: Table 2.1), 72°C for 30 seconds (elongation). HPRT1 was used as reference gene in all RT-qPCR experiments. Relative transcript quantity was determined based on $\Delta\text{Ct}(\text{gene of interest})\Delta\text{Ct}(\text{HPRT1})$ values.

2.2.2 Detection and quantitation of hTERT isoforms by Hydrolysis-probe assisted PCR

TaqMan® Hydrolysis probe-assisted RT-qPCR was used to detect hTERT isoform transcripts. Exon-exon junctions unique for each of hTERT isoforms ($\alpha+\beta$ -hTERT, α -hTERT, β -hTERT, α - β -hTERT) were used as PCR priming sites (Fig. 2.1 B). $\alpha+\beta$ -hTERT are transcript species with α and β site intact, and includes FL-hTERT transcript. Importantly, the TaqMan primers are designed to detect any transcripts with the target junctions, meaning *they do not distinguish between transcripts that are different outside of the PCR-amplified region* (see Fig. 2.1 description). *Additionally, the TaqMan primers do not distinguish recombinant 3xFLAG-hTERT transcript from endogenous hTERT transcript.* FAM-

conjugated DNA probes specific for the hTERT gene were employed to further increase the specificity and sensitivity of the RT-qPCR (Fig. 2.1 B). 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 200nM forward and reverse primers, and 250nM hTERT specific FAM-probe were included per PCR reaction (20 μ l). For reference, HPRT1 transcript abundance was measured alongside by SensiFAST PCR as outlined above.

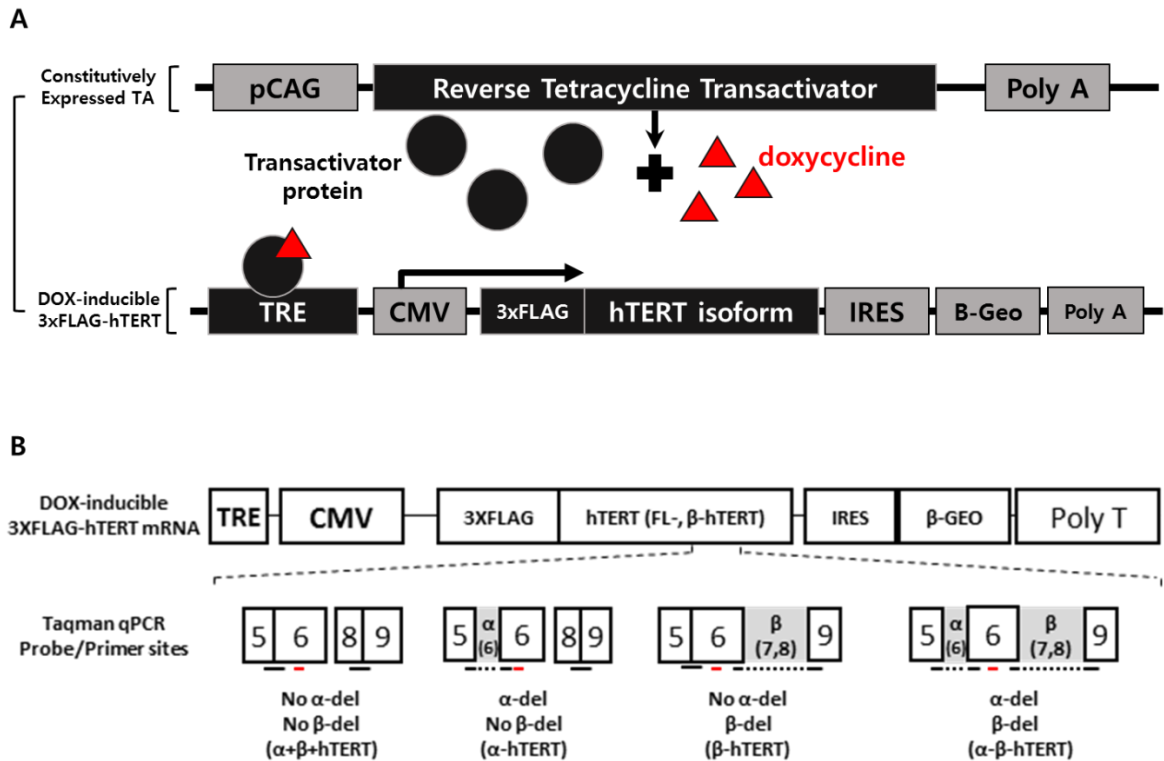


Fig. 2.1 Transgene structure and TaqMan qPCR probe-/primer-binding sites. Dox-inducible overexpression cassette for 3xFLAG-tagged FL-hTERT and 3xFLAG-tagged β-hTERT were cloned and integrated into the genome of HES2 using the PiggyBac Transposon System (vectors donated by Nagy Lab, Mt Sinai Hospital; work of Dr. Jonathan H. Teichroeb). Two transgenes were genomically integrated **A**) PiggyBac rtTA gene for transactivator expression (required for transcription of 3xFLAG-hTERT) and DOX-inducible 3xFLAG-tagged-hTERT expression sequence. **B**) Primers (black) and TaqMan™ probe (red) annealing sites for hTERT variants. *The primers cannot detect the differences in transcripts with alternatively spliced sites outside of the targeted regions. (i.e. α+β+hTERT primers will non-discriminately detect all transcripts with intact α and β sites regardless of alternative splicing that may be present outside of exon5/exon6 junction-to-exon8/exon9 junction region). Moreover, the TaqMan primers do not differentiate between recombinant 3xFLAG-hTERT transcripts and endogenous hTERT transcripts.*

2.2.3 RNA-immunoprecipitation and PCR-assisted detection of TERC

1 x 10⁷ HES2 transgenic clonal cells were trypsinized and lysed in cold non-denaturing buffer (20mM Tris pH8.0, 137mM NaCl, 10% Glycerol, 1% IGEPAL (Sigma), 2mM EDTA, 1X Protease Inhibitor Cocktail Set I (Calbiochem)). The lysate was kept on ice for 30 minutes with constant agitation to help complete lysis. Cell debris was isolated and removed by centrifugation at 800g for 10 minutes. The cleared lysate was co-incubated with Anti-FLAG M2 Magnetic beads (30µl packed volume; Sigma), which was precleared with salmon sperm DNA (10 µg/ml) to prevent non-specific binding of nucleotides to the beads. The incubation was done at 4°C on a rotator overnight. Beads were washed 3 times with the lysis buffer and 2 times with TBE buffer (89mM Tris, 0.5mM EDTA, 89mM boric acid). TRIzol reagent (ThermoFisher) was added to the washed beads to extract bound RNA from 3xFLAG-hTERT proteins. TERC transcript abundance was quantified and compared in doxycycline-negative and doxycycline-positive sample groups.

2.2.4 PCR amplification of mitochondrial DNA for mitochondria DNA copy number

A set of PCR primers (appendix A1: Table 2.1) targeting a small segment of mitochondrial DNA (tRNA^{Leu}) was used to estimate the relative mitochondrial DNA copy number in the sample groups (appendix A1: Table 2.1). A Mammalian Genomic DNA Miniprep Kit (Sigma) was used to extract genomic DNA. Nucleotide content in each sample was measured using a NanoDrop (ThermoFisher) spectrophotometer. Extracted gDNA was preheated to 75°C for 15 minutes to ease the supercoiling of circular mitochondrial DNA. The PCR reaction volume consisted of SensiFAST PCR master mix (5µl; Bioline), 10µM forward/reverse primers (0.2µl), 2µl template gDNA (6ng in mass), and 2.8µl RT-qPCR

grade water. PCR Cycling conditions were 95°C for 2mins (DNA polymerase activation; 1cycle), 95°C for 10 seconds (denaturation), 62°C for 10 seconds (annealing), and 72 °C for 20 seconds (synthesis). A single copy nuclear gene, beta-2-microglobulin (B2M) was used as a reference to normalize the data to the number of genomes per sample.

2.3 Telomerase Activity Assay

To confirm the telomerase activity of 3xFLAG-tagged transgenic hTERT, a telomeric repeat amplification protocol (TRAP) assay was performed as previously described [191]. HES2 and 3xFLAG-hTERT-expressing lines were treated with doxycycline (1µg/ml) for 2 days. Cells were harvested by trypsinization and lysed in CHAPS lysis buffer (150mM KCl, 50mM HEPES pH7.4, 0.1% CHAPS). RNaseOUT™ (Recombinant RNase Inhibitor; ThermoFisher) was included in the lysis buffer to prevent the degradation of TERC. To avoid protein degradation from freezing, all downstream steps were performed immediately and the samples were kept on ice. Total protein content in each sample was quantified by DC™ Protein Assay (BioRad). In the initial step of the tandem polymerase reactions, any TERC-bound hTERT species in the lysate would elongate (by telomerase activity) the TS substrate primer (AATCCGTCGAGCAGAGTT) *in vitro* for 30 mins at 25°C. This was followed by heat-activation of DNA polymerase (SensiFAST; Biorline) and simultaneous heat-inactivation of hTERT protein at 95°C for 2 minutes. The *de novo*-synthesized telomeric sequences was amplified using a return primer ACX (GCGCGGCTTACCCTTACCCTTACCCTAACC). The temperature settings for this PCR (32 cycles; predetermined to ensure exponentially amplifying PCR cycle across all sample groups) were 95°C for 10 seconds (denaturation), 58°C for 10 seconds (annealing), 72°C 30 seconds (elongation). An internal control primers

TSNT (AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT) and NT (ATCGCTTCTCGGCCTTTT) were included. TSNT is semi-competitively amplified by the annealing of TS primer and its dedicated return primer NT during PCR. The primary purpose of this is to rule out potential false-negative outcomes resulting from hindered DNA polymerase during PCR. Secondly, consistent amplification of TSNT amplicon (36bp) is indicative of equal PCR reaction efficiency. The finished PCR reaction mix was electrophoresed through a vertical native 12% polyacrylamide gel at 400V for 45 minutes in 1X TBE buffer (89mM Tris, 0.5mM EDTA, 89mM boric acid) to resolve the product bands. The PAGE gel was stained with X1 SYBR Green I (ThermoFisher) nucleic acid dye in TBE for 30 minutes at room temperature. Bands were visualized using a Gel DocTM EZ (BioRad) and UV. The band intensity values of telomeric repeat products were first normalized to TSNT internal control band intensity.

2.4 Immunodetection of 3xFLAG-hTERT isoforms

2.4.1 Western Blot Analysis

HES2 B6 and F12 clones ($n \geq 3$ /group) were grown under feeder-free condition under 2% O₂ and H₂O₂ treatments. 3xFLAG-hTERT overexpression was induced by direct addition of doxycycline (1 μ g/ml) to the culture medium 48-hours prior to cell harvest. Cells were lysed and fractionated using a mitochondria isolation kit per its instructions (Mitochondria Isolation Kit for Cultured Cells; ThermoFisher). Protease Inhibitor Cocktail Set I (Calbiochem) was added to all lysis/resuspension buffers to prevent protein degradation. The isolated mitochondria and nuclei of mitochondria-enriched, and nuclei-enriched samples were examined by Western Blot analysis with antibodies against voltage-dependent anion

channel (VDAC; mitochondria marker), Prohibitin (mitochondria marker), and histone H3 (nuclear marker) (*see* appendix A2: Table 2.2). VDAC and histone H3 were used for normalization. 3xFLAG- hTERT in nuclear and mitochondrial fractions was traced using an anti-FLAG antibody (Sigma) and quantified by densitometry analysis using Image Lab™ (BioRad). All membranes were developed using a VersaDoc Imaging System (BioRad)

2.4.2 Immunocytochemistry

For immunofluorescence microscopy, HES2 B6 and F12 clones were grown in feeder-free conditions on ChamberSlides™ (Nunc), and were treated with H₂O₂ as previously indicated (*see* section 2.1.4). Cells were fixed and permeabilized in 4% formaldehyde (15 minutes) and in 0.25% Triton™ X-100 for (10 minutes), respectively. Cells were washed with phosphate buffered saline three times in between steps. Cells were blocked with 10% bovine serum albumin solution for 45 minutes at 37 °C. Cells were incubated with anti-TOM20 (1:500; rabbit polyclonal; ProteinTech) and anti-FLAG antibody (1:200; mouse monoclonal; Sigma) in 3% BSA solution overnight at 4°C. Lastly, cells were counterstained with NucBlue (nuclear stain; ThermoFisher). The subcellular localization of 3xFLAG-hTERT was investigated by co-localization using a confocal microscope (Zeiss LSM 5 Duo Vario) and 63X oil-immersion objective lens. Image analysis software Zen (Zeiss) and Image J were utilized to analyze sample images.

2.4.3 Cell count analysis for intra-nuclear localization of 3xFLAG-hTERT isoforms

HES2 B6 and F12 clones were grown in feeder-free condition on ChamberSlides™ (Nunc) for 3 days until ~70% confluency was reached. Cells were treated with doxycycline-containing (1 µg/ml) Essential 8 media for 2 days. The culture medium was renewed daily. H₂O₂ was directly added to the media (100 µM final concentration) 2 hours prior to harvest. Field-of-view images of HES2 B6 (n=11) and HES2 FL (n=12) were taken as outlined in section 2.4.2. All images contained either a whole colony or a part of a colony. Only the clear and round nuclei (DAPI stain) were counted. Partial nuclei at the edge of the images were excluded from analysis. The intra-nuclear localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were categorized into an uniform distribution or a peripheral distribution (Fig. 2.2). Nuclei with uniform distribution of 3xFLAG-hTERT was defined as nuclei with two or more nucleoli (dark and hollow space indicated by arrows in Fig. 2.2 left) as delineated by 3xFLAG-hTERT signal (green). Nuclei with peripheral distribution of 3xFLAG-hTERT was defined as nuclei that has only one nucleolus two thirds of whose boundary is near the nuclear membrane (as delineated by DAPI stain; DAPI not shown in Fig. 2.2). Images were first assigned randomized numbers and alphabets by a third-party member. The blinded images were presented to three volunteers from a third party in random order. Peripheral distribution was defined as an intra-nuclear distribution that exhibits only one nucleolus whose boundary is in close proximity with the nuclear boundary. (Fig. 2.2). Uniform distribution was defined as an intra-nuclear distribution that exhibits more than one nucleolus. The percentage of nuclei with peripheral distribution (out of total nuclei count) determined by the volunteers was averaged for statistical analysis.

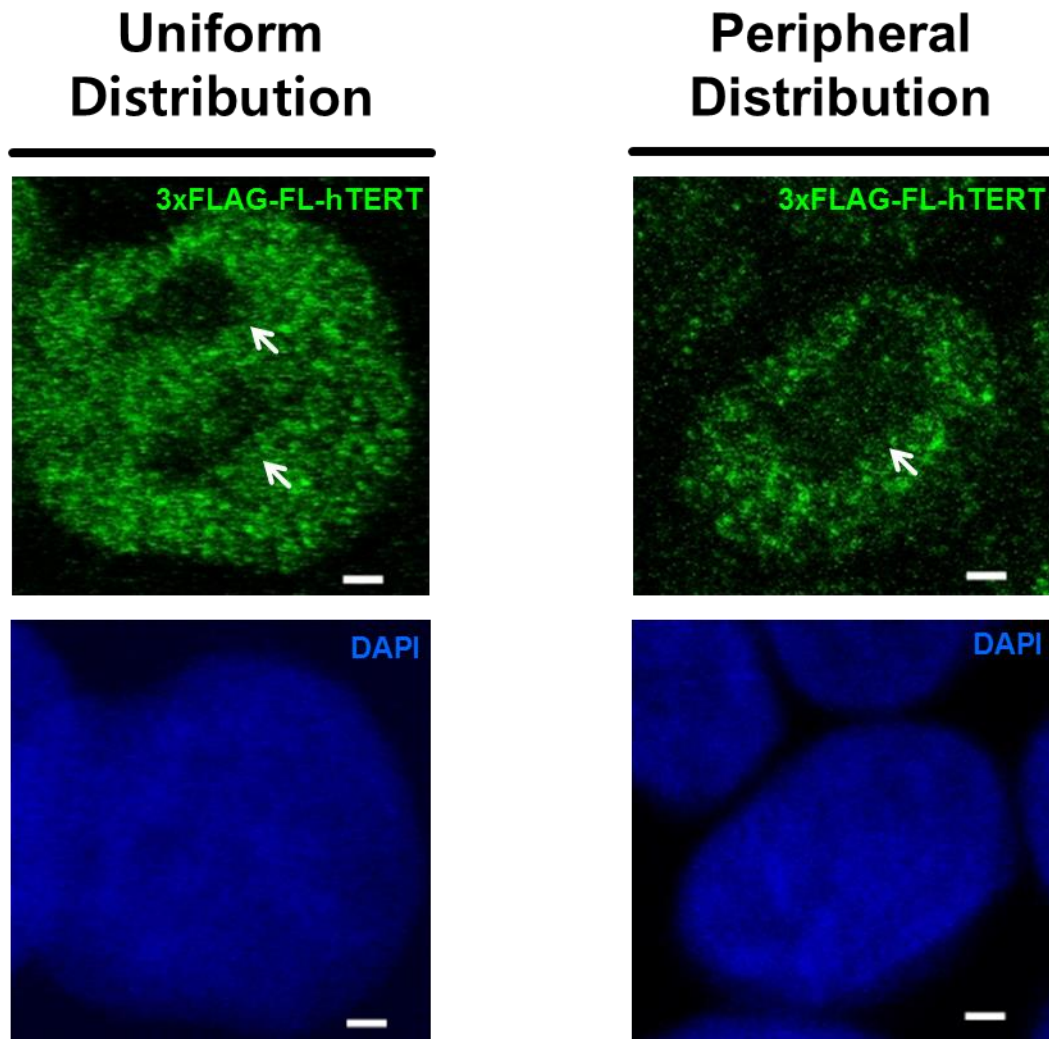


Fig. 2.2: Representative confocal microscopy images of ‘uniform’ and ‘peripheral’ intra-nuclear distribution of 3xFLAG-FL-hTERT within *nuclei*. The intranuclear distribution of 3xFLAG-FL-hTERT was visualized with anti-FLAG antibody and Alexa Fluor 488 secondary antibody (green). DAPI counter stain was used to mark nuclei. Nucleoli are indicated with white arrows. Scales bars (white) = 2 μ m.

2.5 Flow Cytometry

2.5.1 Mitochondria Membrane Potential

The cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide; Sigma) [192, 193] is widely used for microscopic and cytometric estimation and measurement of mitochondrial membrane potential ($\Delta\psi_m$). Aggregates of the dye (J-aggregate) form at the high concentrations reached in energized mitochondria of cells exposed to near-micromolar external concentrations of the dye. J-aggregates are spectrally distinguishable from dye monomers. JC-1 is advantageous over other types of cationic $\Delta\psi_m$ dyes in that it selectively accumulates in mitochondria. JC-1 is excited at 488nm with the highest efficiency. When excited at 488 nm, JC-1 monomers emit green fluorescence with a maximum at 530 nm (green), whereas J-aggregates emit orange-red fluorescence with a maximum at 595 nm (orange-red). A subset of HES2, B6, and FL12 clones were treated with 1 μ g/ml doxycycline for 2 days. All cells were treated with 100 μ M H₂O₂ for 2 hours to induce oxidative/apoptotic stress. Thirty minutes prior to the harvest, cells were treated with JC-1 (10 μ g/ml; dissolved in DMSO) for 30 minutes in the incubator. Cells were washed with PBS (Ca²⁺ free) once and collected by trypsinizing/centrifugation at 300g for 5 minutes. Cell pellets were re-suspended in flow-cytometry staining buffer (Ca²⁺ free PBS, 0.5% BSA). Spectral readings were taken immediately with BD AccuriTM C6 using a blue laser (488nm), bandpass filters FL2 (586 \pm 20nm; J-aggregate) and FL1 (530 \pm 15nm; JC-1 monomer). Twenty thousand events were recorded per group at all channels. 20 μ M Carbonyl cyanide 4-l (trifluoromethoxy) phenylhydrazone (FCCP; chemical dissipater of $\Delta\psi_m$; Molecular Probes®)-treated samples were included as a positive control to represent a depolarized membrane. MMP variation was deduced by calculating FL2 (J-aggregate; high $\Delta\psi_m$) to FL1 ratio (JC-1 monomer; low $\Delta\psi_m$).

2.5.2 Detection of apoptosis

To assess the pro-survival effect of overexpressed 3xFLAG-hTERT in HES2, cells were stained with Annexin V-FITC (ThermoFisher)/7-amino-actinomycin (7-AAD; eBioscience), following 100 μ M H₂O₂ treatment. Annexin V/7-AAD staining provides a way to discriminate between early apoptosis and late apoptosis. When a cell undergoes apoptotic cell death, phosphatidyl serine (PS; normally on the inner leaflet of plasma membrane) becomes exposed on the outer leaflet of the plasma membrane. Annexin V binds PS with high affinity and marks early-apoptotic cells. 7-AAD is a DNA intercalating dye that can enter dead cells with compromised membrane integrity. A subset of HES2, B6, and FL12 clones were treated with 1 μ g/ml doxycycline for 2 days. All cells were treated with 100 μ M H₂O₂ for 2 hours to induce oxidative/apoptotic stress. Following cell harvest by trypsinization/centrifugation (as outlined above), cells were re-suspended in Annexin V-staining buffer (10mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) to 10⁶ cells per/ml. Annexin V-FITC (15 minutes, at room temperature) and 7-AAD (5 minutes, at room temperature) were added to a 100 μ l aliquot of the resuspension. Spectral readings were taken immediately with BD AccuriTM C6 using a blue laser (488nm), bandpass filters FL3 (670 nm longpass; 7-AAD) and FL1 (530 \pm 15nm; FITC Annexin V-FITC). A total of 30000 events were recorded per group at both channels.

2.6 Statistical Analyses

All statistical analyses were performed using Prism 6 (Graph Pad Inc.), and are presented in mean \pm SEM. An unpaired t-test was used for comparisons between DOX-treated and non-DOX-treated samples. Paired t-tests were used for flow cytometer analysis of DOX-

and non-DOX- groups. An One-Way ANOVA with Tukey's post hoc test was used for analyses involving more than two hTERT isoforms or HES2 lines. Statistically significant data are indicated with asterisks (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$). Non-significant results were indicated with 'NS'.

Chapter 3: Results

3.1 Basic cell characterization of human embryonic stem cells

3.1.1 Characterization of pluripotency markers in human embryonic stem cells

Human embryonic stem cells (HES2 cell line) grow as flat and compact round colonies indicative of the primed pluripotent stem cell state [194] (Fig. 3.1 A). Assessed by immunofluorescence microscopy, HES2 cells were positive for pluripotency markers SSEA-4 and OCT-4, but negative for SSEA-1 (Fig. 3.1 C). Transcripts of the $\alpha+\beta$ -hTERT isoform (includes FL-hTERT transcript) and the most prominent hTERT splice variants (β -, α -, α - β -) were detected in HES2 cells that had been cultured under 21% oxygen tension (Fig. 3.1 B). Both β -hTERT and $\alpha+\beta$ -hTERT transcript levels were detected at about 40% of hTERT variant pool measured by TaqMan RT-qPCR (Fig. 2.1), and were significantly higher in abundance (~5-10 fold higher) than α -hTERT and α - β -hTERT transcript levels (Fig. 3.1 B).

3.1.2 Characterization of hTERT isoforms in human embryonic stem cells

HES2 cells were grown under 2% oxygen conditions, with and without doxycycline (DOX) and 100 μ M H₂O₂ treatment to assess the potential side-effect of each treatment. The percent (%) proportion of selected hTERT isoforms in each treatment group was investigated by TaqMan RT-qPCR (Fig. 2.1). When cells were grown under 2% oxygen, the transcript composition of $\alpha+\beta$ -hTERT and β -hTERT remained at approximately 40% and dominant over α -hTERT and α - β -hTERT (in comparison to when the cells grew in 21% oxygen; Fig. 3.2 A and E). However, the splicing pattern changed when H₂O₂ or doxycycline was singly added to the culture media (Fig. 3.2 B and C). The proportion of β -hTERT transcript level

were increased in relation to $\alpha+\beta$ -hTERT and other isoforms when doxycycline and H_2O_2 treatments were applied individually (Fig. 3.2 B and C). In case of doxycycline treatment, the percentage of α - β -hTERT transcript decreased slightly (statistical significance; Fig. 3.2 E). A similar decrease in α - β -hTERT transcript levels were also observed when both H_2O_2 and doxycycline were applied in combination. Neither oxygen tension nor different combinations of doxycycline and H_2O_2 resulted in significant change in any hTERT isoform transcripts except in α - β -hTERT (Fig. 3.2 E). Interestingly, the difference between % $\alpha+\beta$ -hTERT and % β -hTERT was not observed when DOX and H_2O_2 were applied simultaneously (Fig. 3.2 D). In all circumstances, β -hTERT was the most abundant hTERT transcript species expressed in the HES2 hESC line.

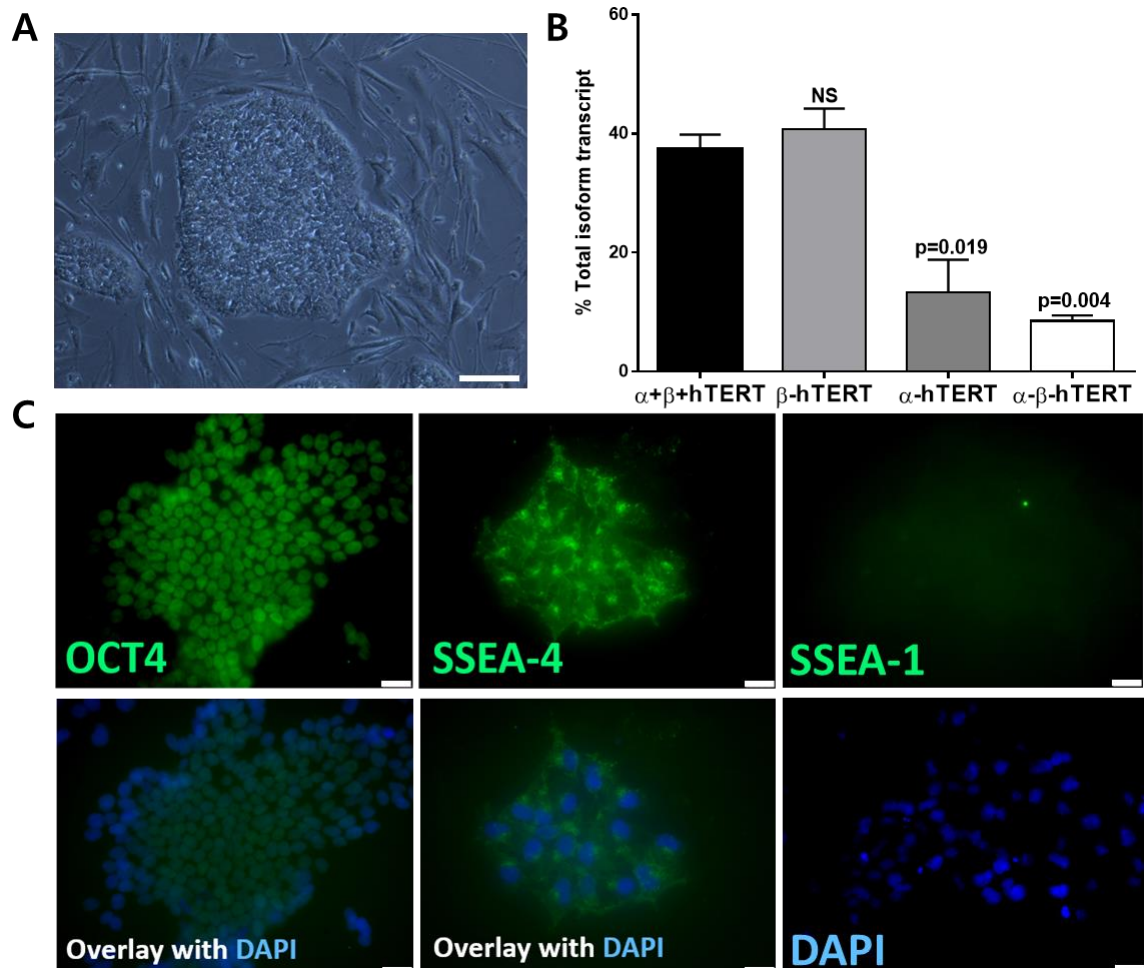


Fig. 3.1: HES2 is a hESC cell line that expresses various markers of pluripotency and different proportions of endogenous telomerase reverse transcriptase (TERT) isoform transcripts. **A)** HES2 (Non-transgenic) hESC colony grown on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells. Scale bar (white) = 200 μ m **B)** HES2 was cultured under ambient oxygen tension ($\sim 21\%$ O_2) and was harvested for RT-qPCR analyses. Primers specific for four different hTERT isoforms ($\alpha+\beta+$, $\beta-$, $\alpha-$, $\alpha-\beta-$) were used to examine TERT isoform composition ($n=4$, error bars = \pm SEM). **C)** HES2 was cultured under 2% O_2 in MEF-free environment to sub-confluency. Cells were fixed and assayed by immunofluorescence microscopy for OCT4, SSEA-4 (positive pluripotency markers), and SSEA-1 (negative pluripotency marker). Cells were counter-stained with DAPI. Scale Bars (white) = 25 μ m. NS = non-significant.

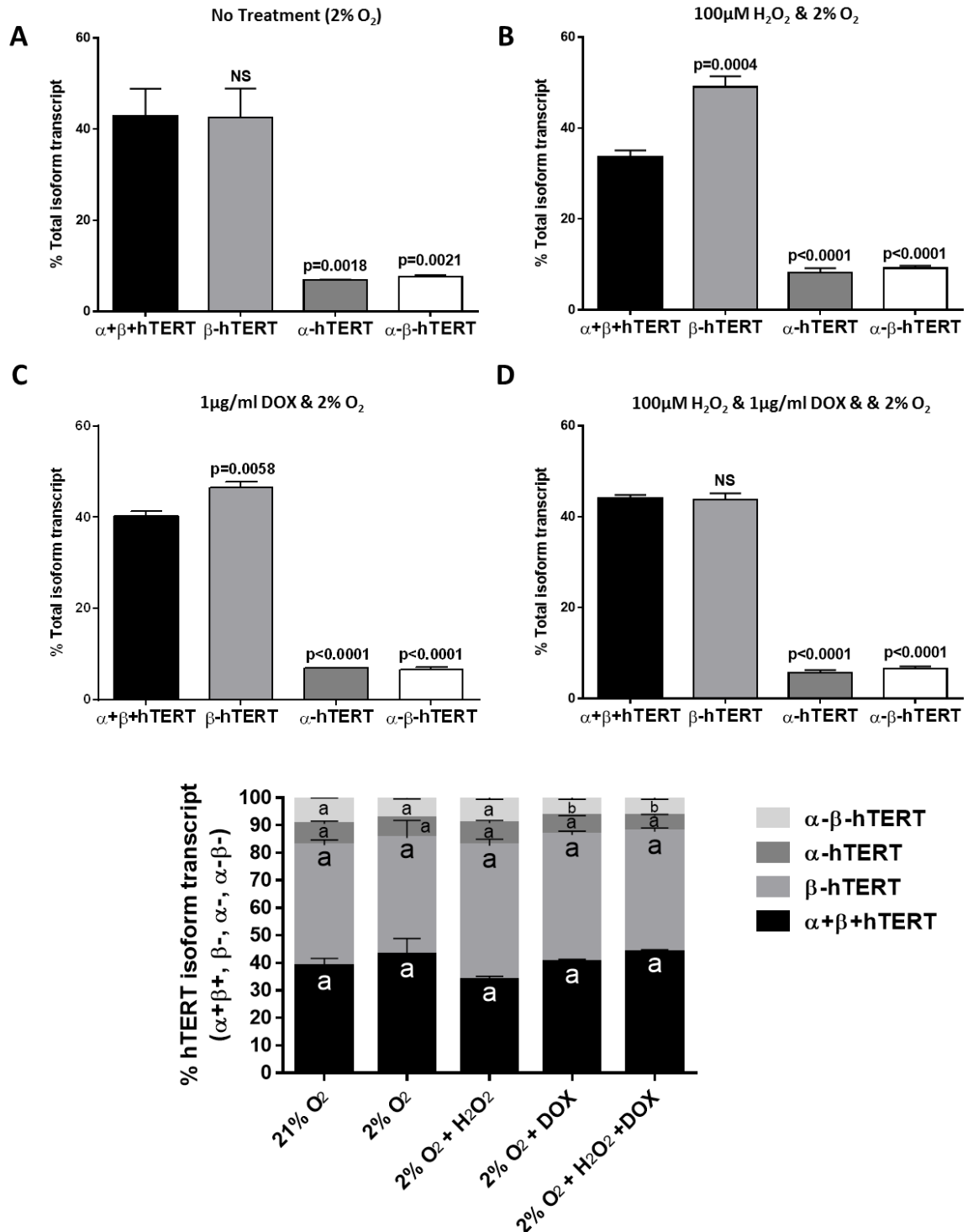


Fig. 3.2: Endogenous β -hTERT transcript is ubiquitously present at high levels in human embryonic cells grown under low oxygen tension conditions, with and without H₂O₂ and/or doxycycline (DOX) treatments. hESCs (HES2 cell line) were grown under low oxygen conditions (2% O₂) with **A)** no treatment, **B)** 100 μ M H₂O₂, **C)** 1 μ g/ml DOX, **D)** 100 μ M H₂O₂ and 1 μ g/ml DOX, **E)** Combined representation of %hTERT isoform change under conditions specified in A-D. Endogenous hTERT variant transcript levels were examined by RT-qPCR (n=3, error bars = \pm SEM). NS = non-significant.

3.2 Characterization of 3xFLAG-hTERT transgenic clones

3.2.1 Characterization of 3xFLAG-hTERT transgene expression level in transgenic hESC clones

DOX-induced 3xFLAG-hTERT isoform expression was examined in a broad panel of HES2 clones by Western blot analysis to select the number of lines for closer examination based on their transgene expression levels. Four HES2 clonal hESC lines overexpressing 3xFLAG- β -hTERT (B2, B3, B6, B7) and four HES2 clones overexpressing 3xFLAG-FL-hTERT (FL7, FL9, FL10, FL12) were randomly chosen and analyzed for protein expression (appendix A3: Fig. 3.1). Based on the preliminary result of 3xFLAG-hTERT induction 12-hours post doxycycline, the selection was further narrowed down to the least expressive β -clone HES2 B3, the most expressive HES2 B6, a FL-clone with similar level of protein induction as B6 HES2 FL9, and the most expressive FL-clone HES2 FL12. HES2 B3 and HES2 B6 hESC lines exhibited the highest levels of 3xFLAG- β -hTERT transcripts. HES2 FL7 and HES2 FL12 hESC clonal cell lines were the least and the most expressive clones for 3xFLAG-FL-hTERT, respectively (appendix A3: Fig 3.1). 3xFLAG-hTERT expression in the selected HES2 hESC clones (B3, B6, FL9, FL12) were examined more closely over a doxycycline time course via RT-qPCR (n=1) and Western blot analysis (Fig. 3.3). Clones B3 and B6 had similar transgene expression at both the transcriptional and translation level. B3 and B6 clones, however, showed much lower transgenic steady-state protein expression levels than FL9 and FL12 hESC clones. Of all, FL12 exhibited the highest FLAG-tagged protein levels at all DOX treatment times. Clone FL9 had markedly lower protein levels than the FL12 clonal hESC line (Fig.3.3 A and C).

3.2.2 Characterization of Pluripotency in HES2 B6 and HES2 FL12 hTERT cell lines

HES2 B6 and FL12 were treated with DOX (2 days; 1 $\mu\text{g}/\text{ml}$) and with H_2O_2 (final 4 hours; 100 μM) to assess the potential side effect of DOX and H_2O_2 treatment on pluripotency marker expression. Positive markers of pluripotency (OCT-4, SSEA-4) were present under all treatment conditions, while the negative marker of pluripotency (SSEA-1) remained absent (Fig. 3.4 and Fig. 3.5).

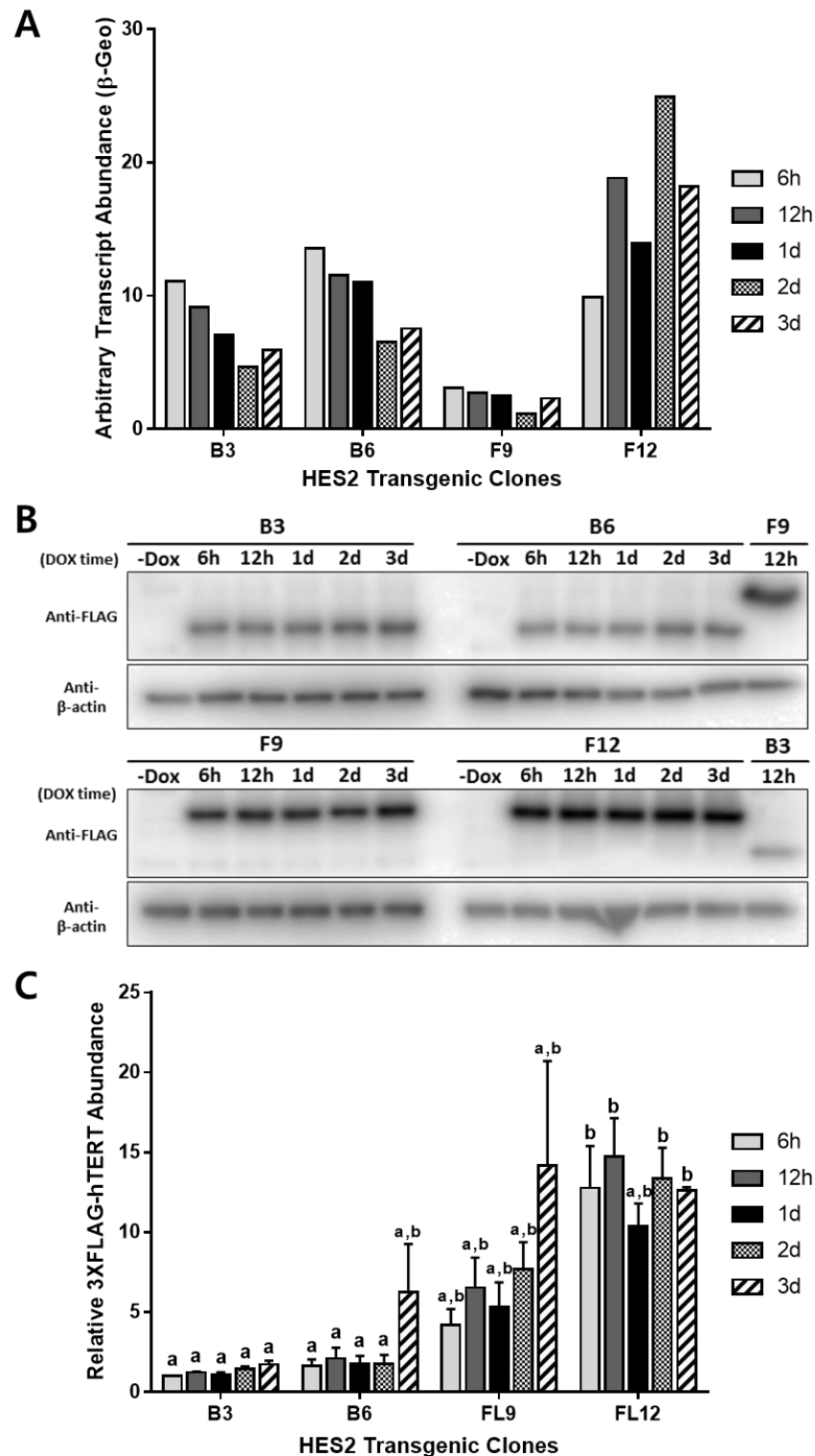


Fig. 3.3: RT-qPCR and Western blot analysis of doxycycline-induced expression of 3xFLAG-hTERT isoforms in HES2 clones over 3-day time course. HES2 clones (HES2 B3 and B6 for DOX-inducible 3xFLAG-β-hTERT, and HES2 FL9 and FL12 for DOX-inducible 3xFLAG-FL-hTERT) were cultured under 2% O₂ and were treated with varying times (h = hours, d = days) of doxycycline (DOX) prior to harvest. **A**) In a pilot study, the abundance of various 3xFLAG-hTERT isoform transcripts was examined by RT-qPCR (n=1) **B**) Western blot using anti-FLAG antibody. **C**) Densitometry analysis was performed for quantification of the proteins. Data was normalized to β-actin (n=3, error bars = ±SEM).

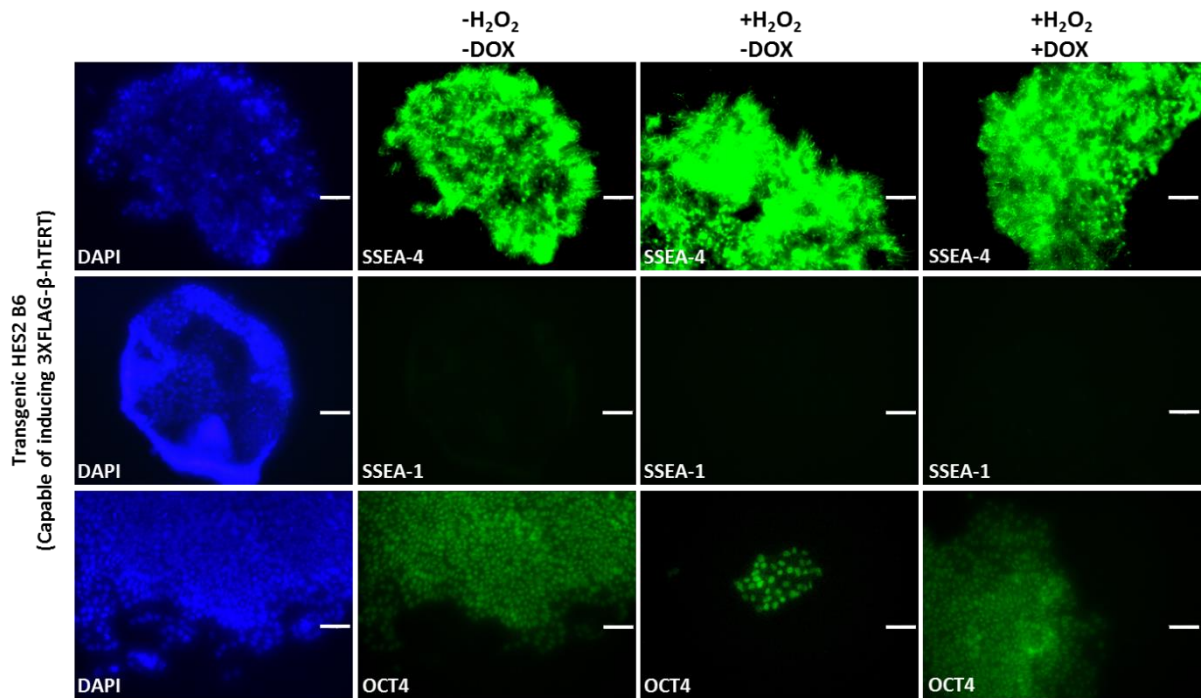


Fig. 3.4: H_2O_2 and DOX treatments do not affect pluripotency marker expression in the 3xFLAG- β -hTERT transgenic HES2 B6 line. HES2 B6 (transgenic hESC line with DOX-inducible 3xFLAG- β -hTERT gene) was cultured in feeder free conditions under 2% O_2 . Cells were treated with or without DOX (2 days; 1 μ g/ml) and H_2O_2 (4 hours; 100 μ M) prior to fixation. Cells were stained for positive markers of pluripotency (OCT4 & SSEA-4; green fluorescence) and a negative marker of pluripotency (SSEA-1). DAPI (blue fluorescence) was used as counterstain and representative DAPI images are only shown for the first treatment group ($-H_2O_2$, $-DOX$). Scale bars = 60 μ m.

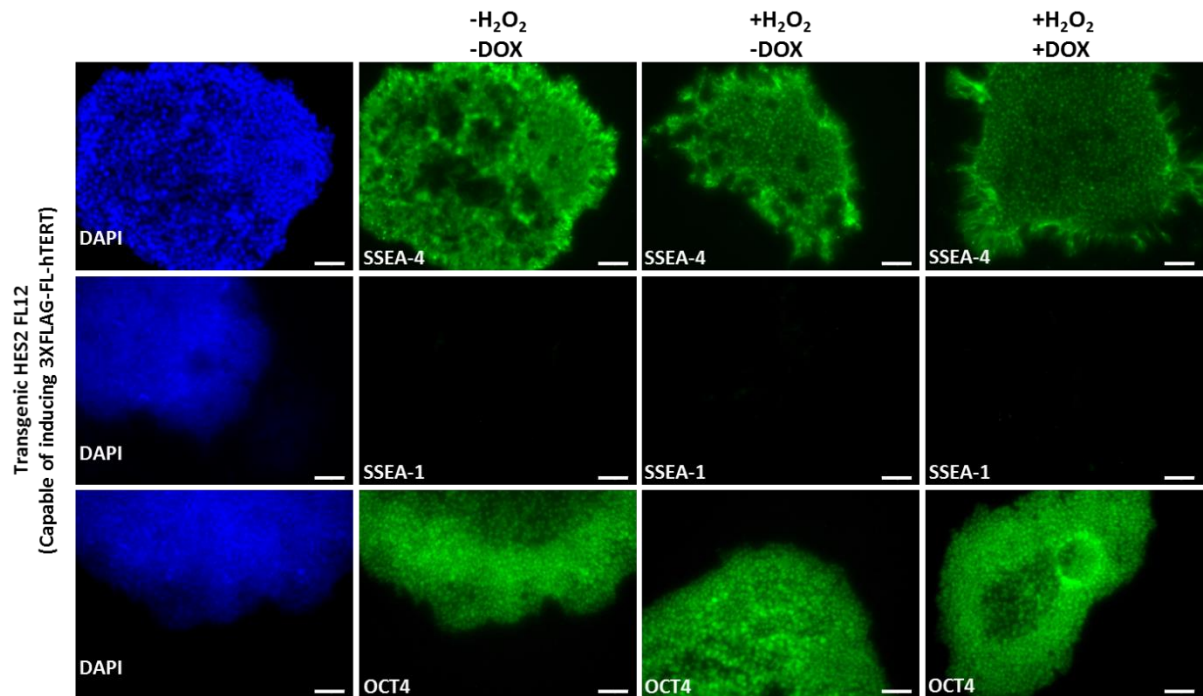


Fig. 3.5: H_2O_2 and DOX treatments do not affect pluripotency of the 3xFLAG-FL-hTERT transgenic HES2 FL12 lines. HES2 FL12 (transgenic hESC line with DOX-inducible 3xFLAG-FL-hTERT gene) were cultured in feeder free condition under 2% O_2 . Cells were treated with or without DOX (2 days; 1 μ g/ml) and H_2O_2 (4 hours; 100 μ M) prior to fixation. Cells were stained for positive markers of pluripotency (OCT4 & SSEA-4; green fluorescence) and a negative marker of pluripotency (SSEA-1). DAPI (blue fluorescence) was used as counterstain and representative DAPI images are only shown for the first treatment group ($-H_2O_2$, $-DOX$). Scale bars = 60 μ m.

3.3 Characterization of DOX-induced recombinant hTERT in HES2 B6 and HES2 FL12

3.3.1 Endogenous TERC-binding of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT subunits

RNA-immunoprecipitation (RIP) using a FLAG antibody was performed to determine whether the 3xFLAG-hTERT transgenic proteins have the ability to bind to the telomerase RNA-component (TERC). TERC recovery from the RIP eluents of DOX-treated samples and non-DOX-treated samples were compared. Overexpression of 3xFLAG-hTERT by doxycycline prior to RIP resulted in approximately 60-fold increase in TERC recovery for 3xFLAG-FL-hTERT, and 10-fold increase for 3xFLAG- β -hTERT (Fig. 3.6 A, B, C). Doxycycline-treated HES2 (non-transgenic; negative control) did not result in any change in TERC recovery, and resulted in a very low TERC PCR-amplification (Fig. 3.6 D).

3.3.2 Endogenous RMRP-binding of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT subunits

Another RIP using a FLAG antibody was performed to determine whether the 3xFLAG-hTERT transgenic proteins have the ability to bind to the RNA component of RNase MRP (RMRP). Overexpression of 3xFLAG-hTERT by doxycycline prior to RIP resulted in an approximately 4-fold increase in RMRP recovery for 3xFLAG-FL-hTERT, and a 2-fold increase for 3xFLAG- β -hTERT (Fig. 3.7 A, B, C). RMRP recovery from DOX-treated HES2 (non-transgenic; negative control) was not different from its control. Notably, the amount of PCR-amplified RMRP from HES2 control group (Fig. 3.7 D) was significant and was comparable to that from 3xFLAG- β -hTERT RNA immunoprecipitates (Fig. 3.7 B).

3.3.3 Telomerase activity in HES2 B6 and HES2 FL12 hESCs

The effect of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT overexpression on total telomerase activity of HES2 B6 and FL12 cell lines was investigated by the telomerase repeat amplification protocol (TRAP) assay (Fig. 3.8). Negative controls (heat-inactivated lysate and no-lysate control) did not generate any product bands (Fig.3.8 A). Doxycycline in non-transgenic HES2 had no effect on the endogenous telomerase activity (Fig. 3.8 B top). DOX-induction of recombinant β -hTERT in HES2 B6 resulted in ~30% decrease in the total telomerase activity, whereas DOX-induction of recombinant FL-hTERT in HES2 FL12 had no effect (Fig. 3.8 B bottom left and right, respectively).

3.3.4 Re-visiting DOX-induction of 3xFLAG-hTERT transcription in HES2 clones

Since overexpression of 3xFLAG-FL-hTERT did not result in an the expected increase in telomerase activity in HES2 FL12 (Fig. 3.8 B), we re-investigated the 3xFLAG-hTERT transgene expressions and endogenous hTERT isoform transcript abundance in the transgenic HES2 clones. The transcript level of different hTERT isoforms in HES2 FL12 and HES2 B6 was examined after DOX-induction by hydrolysis-probe-assisted RT-qPCR. The addition of DOX in HES2 FL12 resulted in ~500-fold increase in FL-hTERT transcript, but surprisingly a ~300-fold increase in β -hTERT transcript, and ~200-fold increase in α - β -hTERT transcript was also observed compared to non-induced controls (Fig.3.9 A). Interestingly, the HES2 B6 cell line expressed only the β -deletion-containing TERT isoforms upon doxycycline treatment. β -hTERT abundance rose sharply (~1000-fold in comparison with its non-induced control) after doxycycline treatment in HES2 B6. A ~150-fold increase in α - β -hTERT was also observed (Fig.3.9 B).

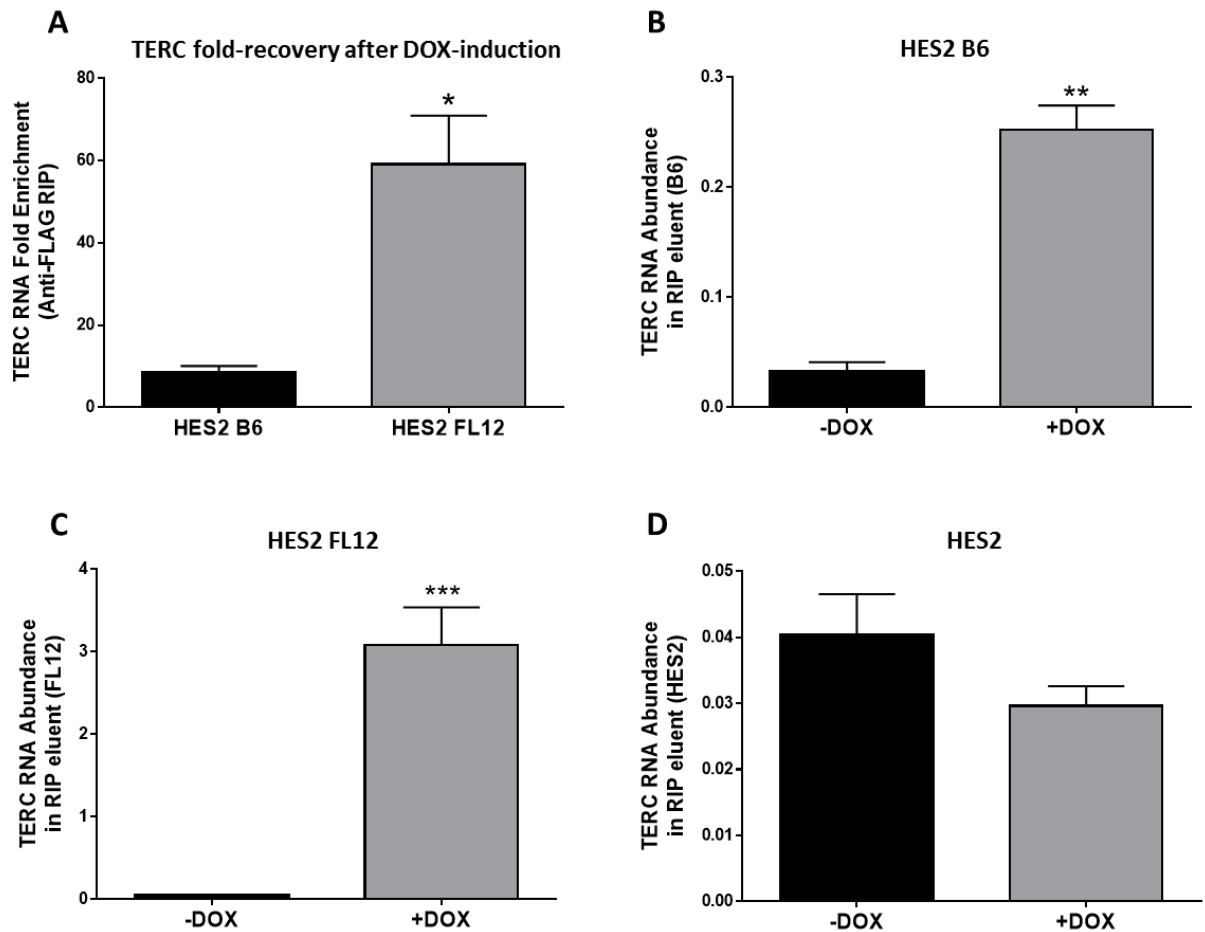


Fig. 3.6: Doxycycline-induced transgenic telomerase proteins (3xFLAG- β -hTERT and 3xFLAG-FL-hTERT) bind to the telomerase RNA component (TERC). Cells were grown under 2% O₂ and were treated with 1 μ g/ml doxycycline (DOX) for two days prior to harvest. DOX-induced 3xFLAG-hTERT isoforms were isolated from their respective clones by immunoprecipitation using anti-FLAG-magnetic beads. Eluted proteins were subsequently denatured to release any bound RNA for downstream RT-qPCR analysis. **A)** Comparison of results in HES2 transgenic lines, and individual TERC enrichment data from **B)** HES2 B6 **C)** HES2 FL12 **D)** Non-transgenic HES2 control (n=3, error bar= \pm SEM, **= P <0.01, ***= P <0.001).

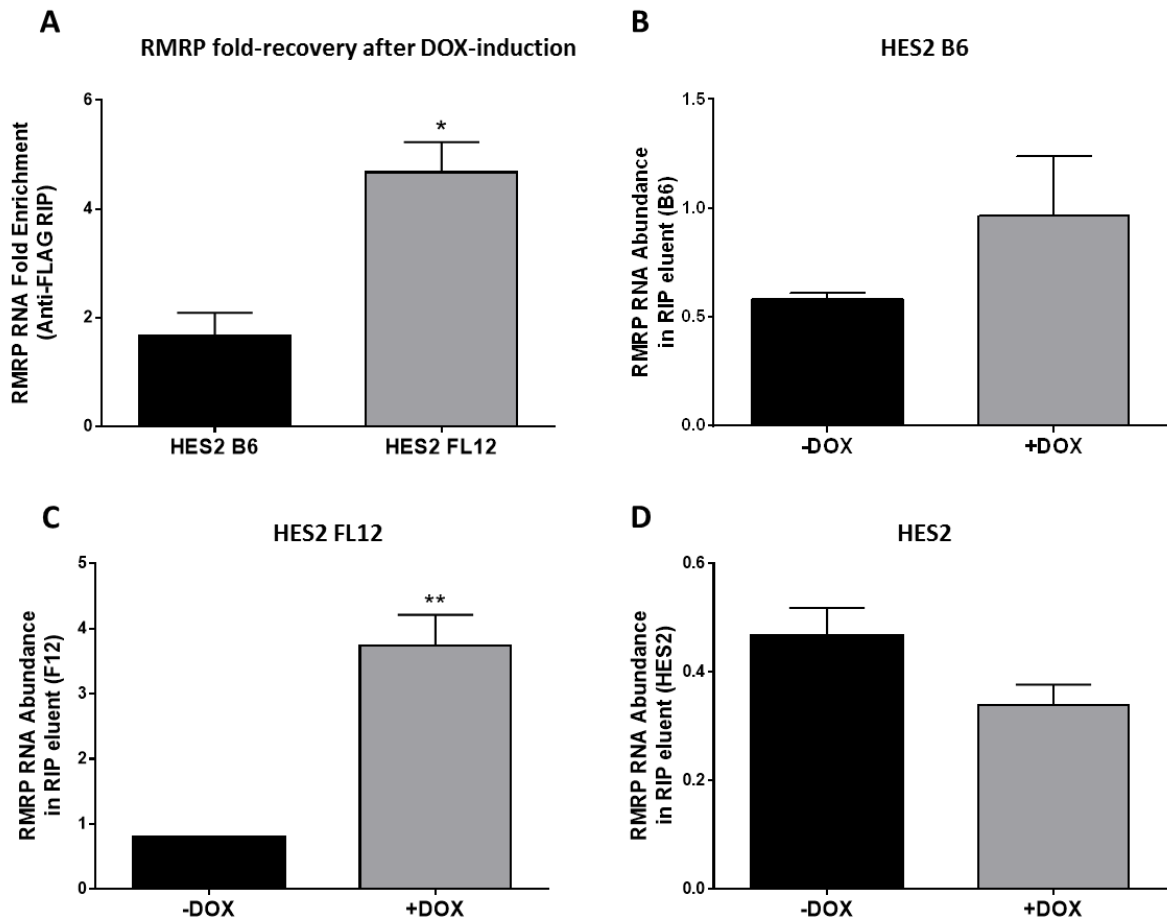


Fig. 3.7: Enrichment of RNA component of mitochondrial RNA processing endoribonuclease (RMRP by doxycycline-induced transgenic telomerase proteins (3xFLAG- β -hTERT and 3xFLAG-FL-hTERT). Cells were grown under 2% O₂ and were treated with 1 μ g/ml doxycycline (DOX) for two days prior to harvest. DOX-induced 3XFLAG-hTERT isoforms were isolated from their respective clones by immunoprecipitation using anti-FLAG-magnetic beads. Eluted proteins were subsequently denatured to release any bound RNA for downstream RT-qPCR analysis. **A)** Comparison of results in HES2 transgenic lines, and individual RMRP enrichment data from **B)** HES2 B6 **C)** HES2 FL12 **D)** Non-transgenic HES2 control (n=3, error bar = \pm SEM).

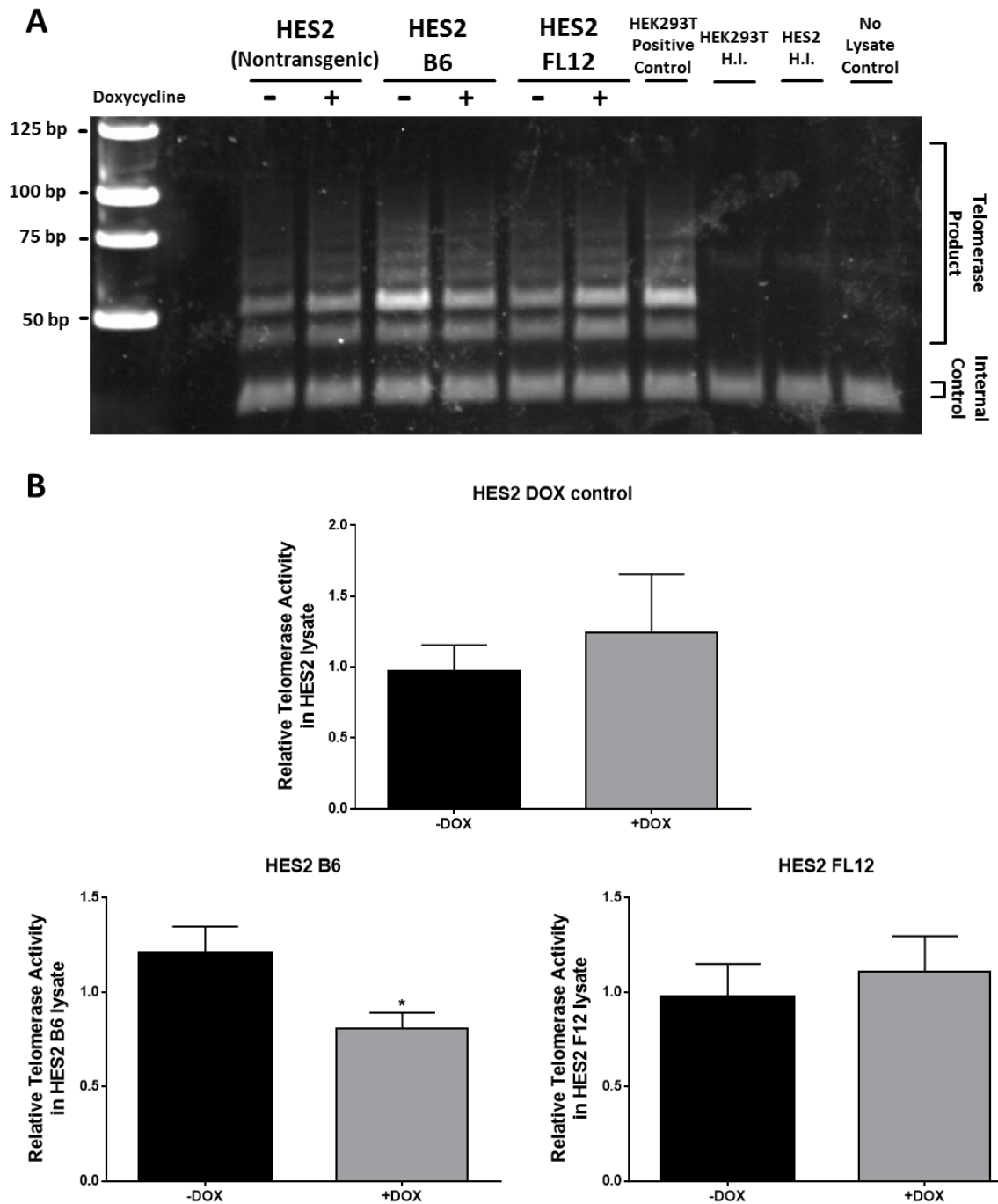


Fig. 3.8: Altered telomerase activity levels in doxycycline (DOX)-induced transgenic telomerase proteins (3xFLAG-hTERT) in hESCs. Cells were grown under 2% O₂ and were treated with 1µg/ml doxycycline for two days prior to harvest. **A)** Representative DNA gel of telomerase-repeat amplification assay by qPCR. Relative telomerase activity between samples is reflected in the intensity and the range of telomerase product bands. H.I. = Heat-Inactivated **B)** Band intensity of amplified telomerase product was measured by densitometry analysis and represented as relative telomerase activity. Data were normalized to internal control band intensities (n=4, error bar=±SEM).

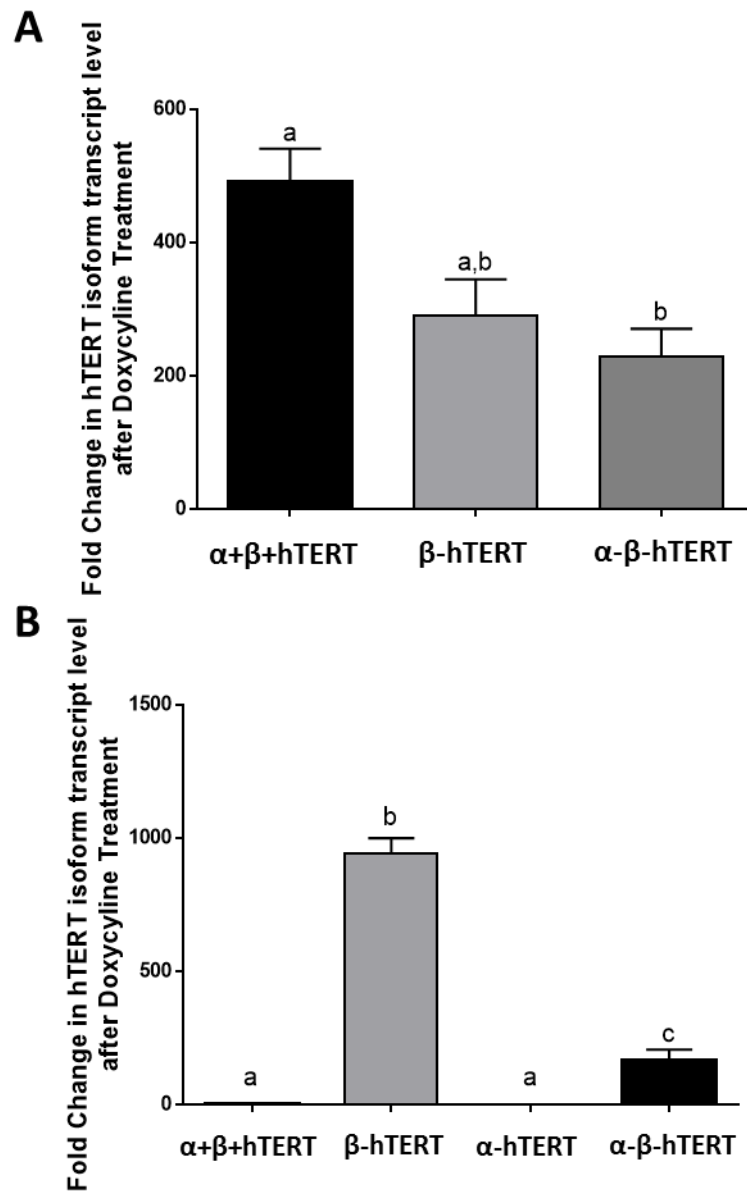


Fig. 3.9: DOX-induction of 3xFLAG-hTERT isoforms results in a simultaneous increase in the amount of other hTERT transcript variants containing β -deletion. A) HES2 FL12 and B) B6 were grown under 2% O_2 and were treated with 1 μ g/ml doxycycline for two days prior to harvest. hTERT variant transcript abundance in each sample was examined by RT-qPCR using primer sets specific for $\alpha+\beta+$, $\alpha-$, $\beta-$, $\alpha-\beta-$. *The primers do not discriminate endogenous hTERT species from transgenic hTERT species.* n=3, error bars = \pm SEM.

3.4 3xFLAG-hTERT localization under H₂O₂-induced oxidative stress

3.4.1 Sub-cellular Western Blot Analysis of 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT expressing hESC lines

H₂O₂ (100μM) was applied to promote mitochondrial localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in the respective transgenic cell lines. Cells were fractionated into nuclear fraction and mitochondrial fraction for protein analysis. Western blot analysis of sub-cellular fractions demonstrated that the level of mitochondrial 3xFLAG-β-hTERT appears to increase with 1h H₂O₂ (100μM) treatment, but fluctuates with longer H₂O₂ exposure (Fig. 3.10 Column A). However, no statistical significance (p>0.05) was observed between different treatment groups. Similarly, 3xFLAG-FL-hTERT levels in the mitochondrial fraction initially increased at 30-minute post H₂O₂ (100μM) treatment, but decreased over longer exposure time (Fig. 3.11 Column A). Again, no statistical significance (p>0.05) was detected between different treatment groups. Similarly, neither the levels of 3xFLAG-β-hTERT nor 3xFLAG-FL-hTERT changed significantly in the nuclear fractions over the H₂O₂ treatment time course (Fig. 3.10 B and Fig. 3.11 B). Additionally, I failed to observe significant intra-cellular movement of 3xFLAG-hTERT when I performed the same fractionation experiment using prohibitin as a mitochondrial marker (appendix A4: Fig. 3.2).

3.4.2 Immunocytochemistry and Image analysis of 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT localization in hESCs

Due to the inconclusive results obtained by the cellular fractionation studies, I monitored 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT cellular localization by immunofluorescence microscopy. I failed to see co-localized signal from MitoTracker (mitochondria) and M2-anti-FLAG/Alexa Fluor 488 (3xFLAG-hTERT) in either HES2

transgenic clones (Fig. 3.12). Much of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT remained in nuclei regardless of different durations of H₂O₂ exposure (Fig. 3.12). Such nuclear localization of 3xFLAG-FL-hTERT persisted even when HES2 FL12 cells were exposed to 21% O₂ for 3 days (appendix A5: Fig.3.3). Interestingly, there appeared to be distinct patterns of 3xFLAG-hTERT species' distribution within the nuclei. Most cells exhibited 'uniform' intra-nuclear distribution of 3xFLAG-hTERT (indicated by the red arrow in Fig. 3.12). Some nuclei exhibited 'peripheral' intra-nuclear distribution of 3xFLAG-hTERT (indicated by the white arrow in Fig. 3.12). The number of cells exhibiting the different intra-nuclear distributions of 3xFLAG-hTERT was counted from immunofluorescence images. However, the addition of doxycycline to HES2 B6 (Fig. 3.13 A) and HES2 FL12 (Fig. 3.13 B) resulted in no significant change in the intra-nuclear localization of 3xFLAG-hTERT.

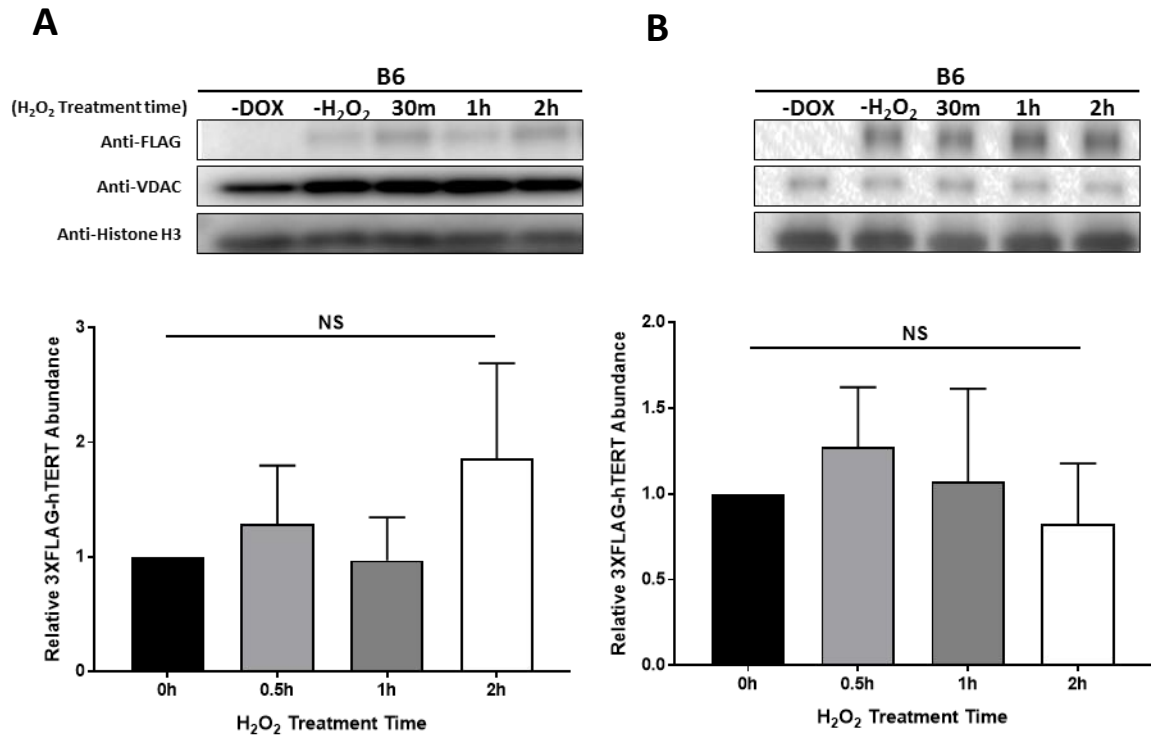


Fig. 3.10: H₂O₂-induced Oxidative stress and its effect on the cellular localization of 3xFLAG- β -hTERT. Cells were grown under 2% O₂ and were treated with 1 μ g/ml DOX for two days prior to harvest. 100 μ M H₂O₂ was applied at different times (30m, 1hr, 2hr) prior to harvest. Nuclei-enriched fraction was isolated via fractionation by differential centrifugation. The amount of 3xFLAG-hTERT species in respective sample groups were investigated by Western blot/densitometry analysis. Results were normalized to VDAC and Histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to H₂O₂ negative control. Western Blot and densitometry analysis plots of **A**) mitochondrial fraction and **B**) nuclear fraction. n=3, error bar= \pm SEM. NS = non-significant

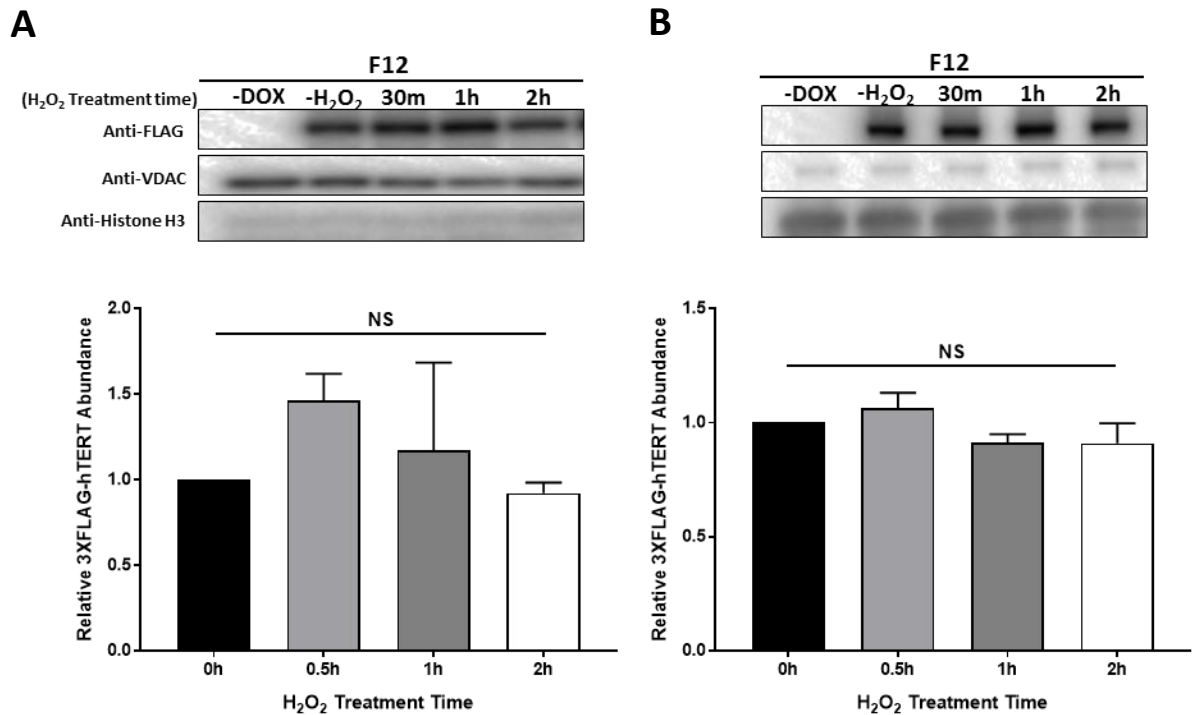


Fig. 3.11: H₂O₂-induced Oxidative stress and its effect on the cellular localization of 3xFLAG-FL-hTERT. Cells were grown under 2% O₂ and were treated with 1 μg/ml DOX for two days prior to harvest. 100 μM H₂O₂ was applied at different times (30m, 1hr, 2hr) prior to harvest. Nuclei-enriched fraction was isolated via fractionation by differential centrifugation. The amount of 3xFLAG-hTERT species in respective sample groups were investigated by Western blot/densitometry analysis. Results were normalized to VDAC and Histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to H₂O₂ negative control. Western Blot and densitometry analysis plots of **A**) mitochondrial fraction and **B**) nuclear fraction. n=3, error bar=±SEM. NS = non-significant

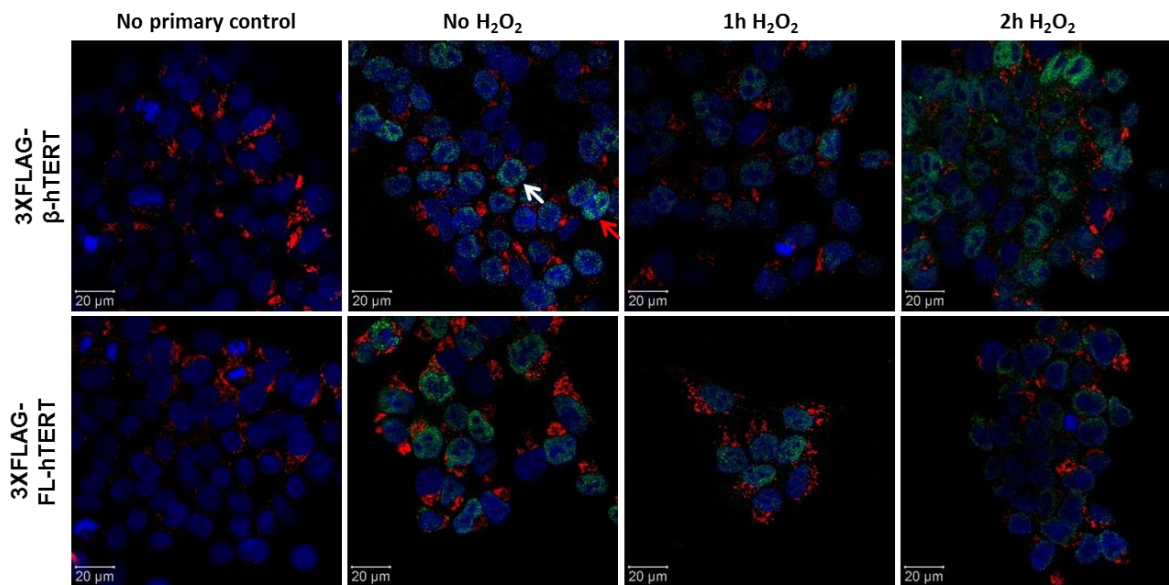


Fig. 3.12: H₂O₂-induced effect on the intra-cellular localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in hESCs. Transgenic hESCs were grown under 2% O₂ and transgenes induced by treated with 1 μg/ml DOX for two days prior to harvest. 100 μM H₂O₂ was applied at different durations (1hr, 2hr) prior to fixation. Immunofluorescence-microscopy images of 3xFLAG-hTERT (green fluorescence), mitochondria (MitoTracker; red fluorescence), and nuclei (DAPI; blue fluorescence) in HES2 transgenic clones. Images were taken using a confocal microscope (63X oil-immersion objective lens, x10 eyepiece lens). 3xFLAG-hTERT were predominantly nuclear, and exhibited either peripheral localization (white arrow; upper row second column) or more uniform distribution within nuclei (red arrow). Scale bar = 20 μm.

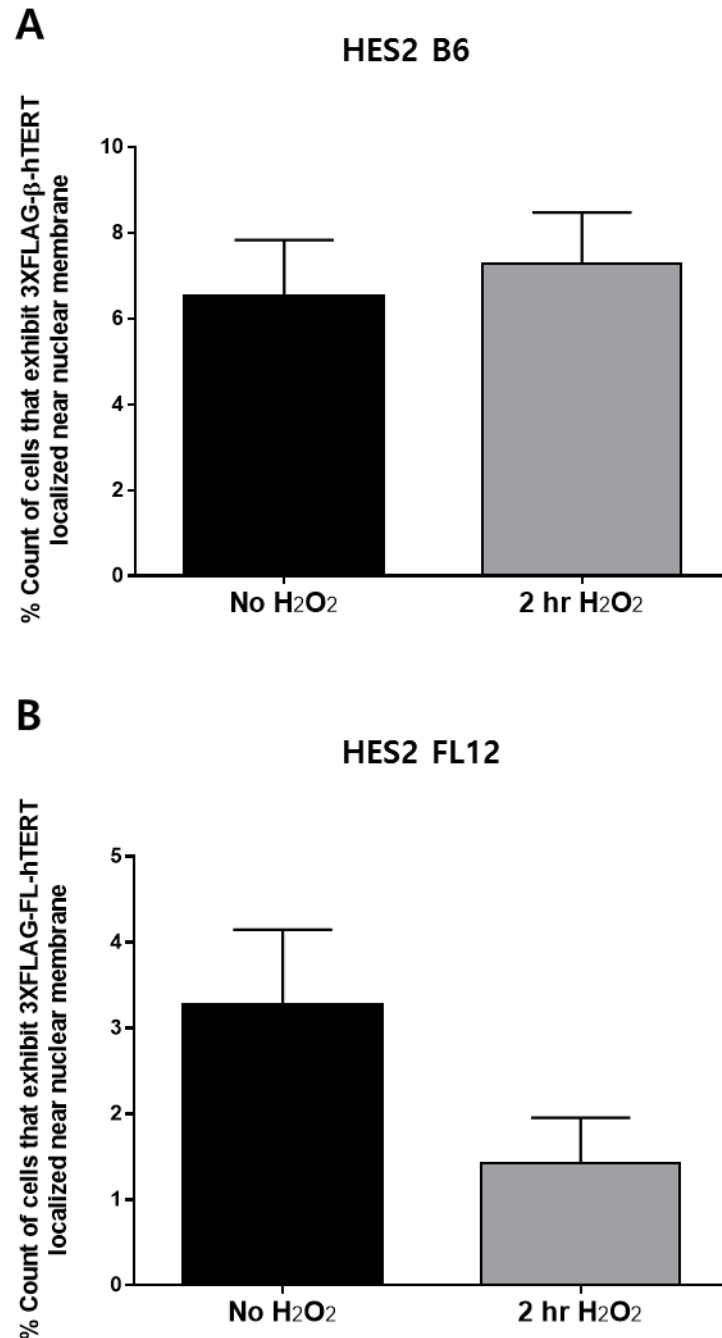


Fig. 3.13: Microscopic analysis of intra-nuclear localization of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT under H₂O₂-induced oxidative stress. Multiple confocal images of HES2 B6 and HES2 FL12 (see Fig. 3.12) were taken and blinded. Percentages of cells that exhibited peripheral 3xFLAG-hTERT localization was counted and averaged by three volunteers. Only the whole and fully visible cells (nuclei) were taken into account. **A)** 3xFLAG- β -hTERT (n=11) **B)** 3xFLAG-FL-hTERT (n=12). Error bars \pm SEM

3.5 The effect of overexpressed 3xFLAG-hTERT on mitochondrial function

3.5.1 The effect of DOX-induced 3xFLAG-hTERT on mitochondria membrane potential

It was previously shown that ectopic expression of FL-hTERT augments mitochondria membrane potential in human embryonic lung fibroblast cells [195]. Human embryonic stem cells (HES2 cell line) were stained with JC-1 dye, which accumulates within the mitochondria and varies its fluorescence excitation in an MMP-dependent manner. The results were examined and analyzed by flow cytometry (Fig. 3.14). Both 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT overexpression in hESCs appeared to slightly decrease MMP activity (2nd and 3rd bars) compared to the doxycycline control group in non-transgenic HES2 cells (1st bar) (Fig. 3.15). However, no significant change in MMP was observed for either transgenic clones.

3.5.2 The effect of DOX-induced 3xFLAG-hTERT on mitochondrial DNA copy number

A previous report has shown that ectopically expressed hTERT results in decreased mitochondrial DNA copy number in human embryonic lung fibroblast cells under 21% oxygen tension [195]. The relative mitochondrial DNA copy number was compared in HES2 B6 and HES2 FL12 clones before and after DOX-induction of 3xFLAG-hTERT species. Approximately a 20% decrease (statistically significant) in mitochondrial DNA copy number was observed when HES2 cells were treated with doxycycline (Fig. 3.16 A). Interestingly, doxycycline treatment of HES2 B6 and HES2 FL12 cells resulted in neither reduction nor increase of mitochondrial DNA copy number (Fig. 3.16 B and C).

3.5.3 The effect of DOX-induced 3xFLAG-hTERT on mitochondria biogenesis and function

Mitochondria biogenesis is regulated by several nuclear genes. A panel of markers for mitochondria biogenesis was monitored by RT-qPCR after doxycycline treatment in HES2 clonal lines (Fig. 3. 17). No significant change in steady-state transcript abundance was observed for any of the examined genes. Notably, POLG (mitochondrial DNA polymerase subunit) did not change with doxycycline treatment in any of the HES2 lines (Fig. 3.17 first bar plot). *SOD2* (mitochondrial anti-oxidant defense enzyme) and *UCP2* (potent mitochondria uncoupler protein) expression levels were also estimated by RT-qPCR. In agreement with previous literatures, *UCP2* transcript was highly abundant in comparison to the biomarkers of mitochondria biogenesis (Figs. 3.17 and 3.18). No significant change was observed with either of these genes following doxycycline treatment.

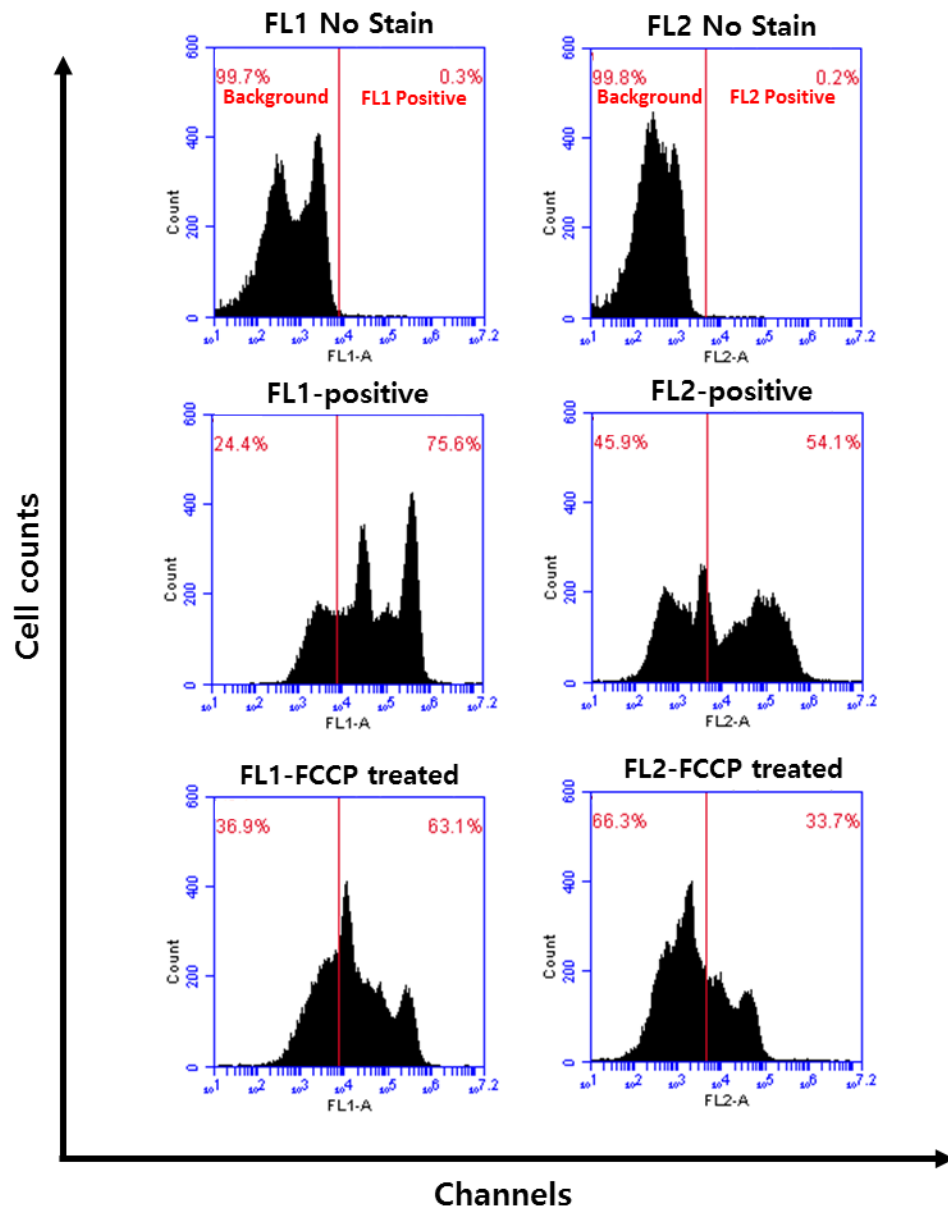


Fig. 3.14: Mitochondrial membrane potential (MMP) gating strategy and controls for flow cytometry experiment with JC-1 dye measurements. 3xFLAG-hTERT isoform expression was induced via 1 μ g/ml DOX (2 days) in HES2 B6 and HES2 FL12. Cells were grown under 2% O₂ and were stained with JC-1 dye for 30 minutes. Control groups were no JC-1 control, 20 μ M FCCP (chemical uncoupler of mitochondria electron transport chain). Mean fluorescence from FL1 and FL2-positive populations were measured for FL2:FL1 ratio calculation. Cell debris was excluded from analysis. Gated-events (15,000) were recorded and analyzed.

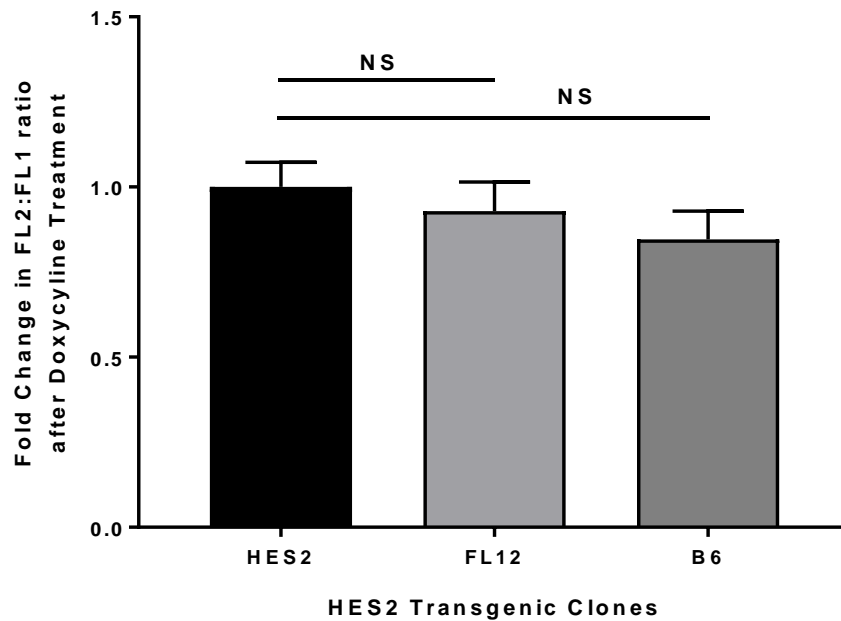


Fig. 3.15: 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT do not affect mitochondrial membrane potential (MMP) in hESCs. Gated mean fluorescence ratio of J-aggregate (FL2; orange-red; bandpass filter $586 \pm 20\text{nM}$) to monomer (FL1; green; bandpass filter $530 \pm 15\text{nM}$) was measured and calculated by flow cytometry. The fold change in FL2:FL1 ratio before and after the addition of doxycycline in HES2, HES2 FL12, HES2 B6 is shown as a bar plot. $n=4$, error bars = $\pm\text{SEM}$, NS = non-significant.

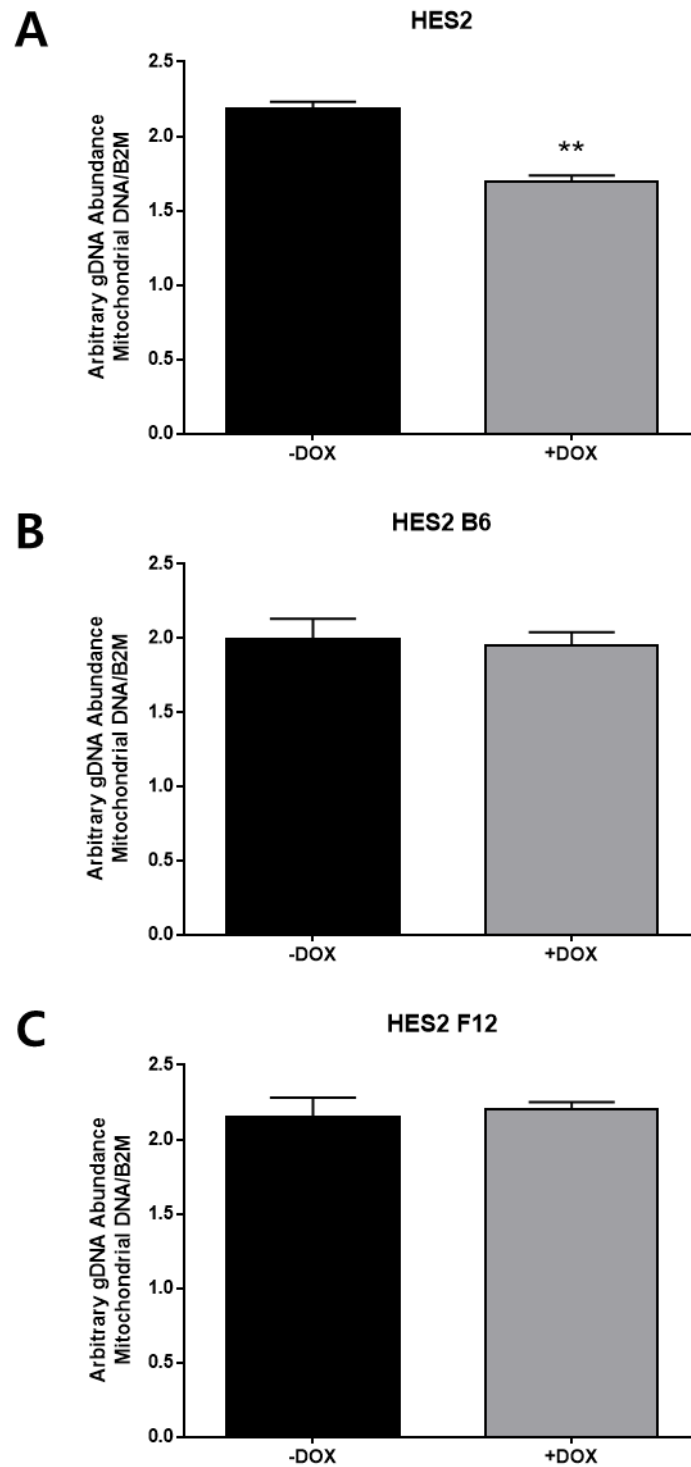


Fig. 3.16: Doxycycline affects mitochondria copy number in hESCs. 3xFLAG-hTERT isoform expression was induced via 1 μ g/ml DOX (2 days) in HES2 B6 and HES2 FL12 clonal cell lines. HES2 (nontransgenic) was also treated with doxycycline (as drug control). All cells were grown under 2% O₂ tension. The mitochondria DNA copy number was estimated by PCR-amplifying a small segment of mitochondria DNA. Results were normalized to B2M (a single copy nuclear gene). A) HES2 B) HES2 B6 C) HES2 FL12 (n=3, error bars = \pm SEM).

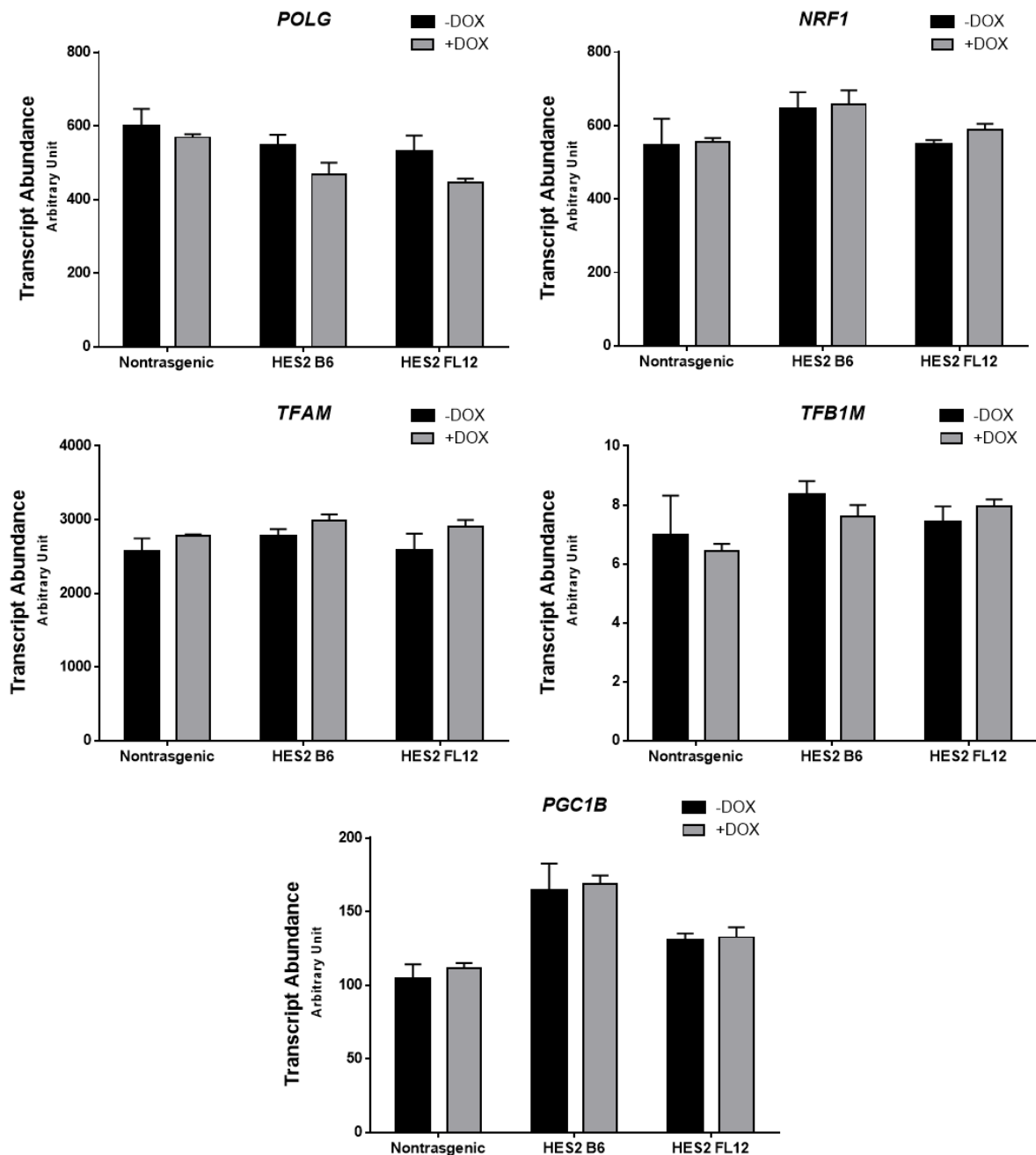


Fig. 3.17: The effect of 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT overexpression on mitochondria biogenesis regulator gene expression. The transcript levels of mitochondria biogenesis regulators were examined by RT-qPCR. HES2, HES2 B6, HES2 FL12 clonal hESC lines were treated with 1 μ g/ml DOX for 2 days prior to analysis. n=3, error bars = \pm SEM

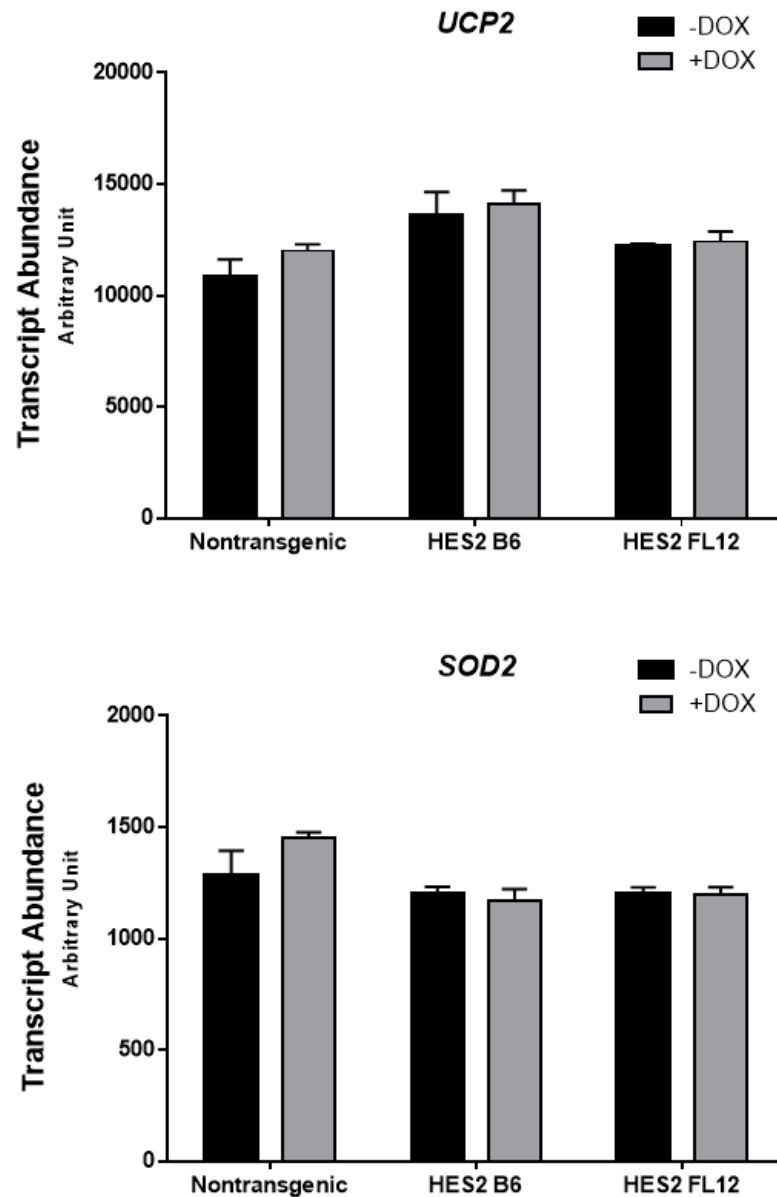


Fig. 3.18: Dox-induced 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT overexpression does not affect UCP2 and SOD2 transcript abundance levels. The transcript levels of *SOD2* (antioxidant enzyme) and *UCP2* (endogenous mitochondrial uncoupler) were examined via RT-qPCR. HES2, HES2 B6, HES2 FL12 clonal hESC lines were treated with 1 μ g/ml DOX for 2 days prior to analysis (n=3, error bars = \pm SEM).

3.6 The effect of doxycycline-induced 3xFLAG-hTERT on cell survival in HES2 lines under H₂O₂-induced oxidative stress

Cell viability, apoptosis, and cell death were measured by flow cytometry. Viable, apoptotic, and dead cell groups were defined as annexin V-FITC⁻/7-AAD⁻, annexin V-FITC⁺/7-AAD⁻, and 7-AAD⁺ populations, respectively. (Fig. 3.19). Doxycycline treatment in HES2 resulted in no change in the assessed cell populations (Fig. 3.20 A, Fig. 3.21 A, and Fig. 3.22 A). The percent viability of HES2 B6 decreased by ~5% following DOX-induction (Fig. 3.20 B). At the same time, the percent apoptotic population of HES2 B6 increased by ~5% following DOX treatment, but the percent dead cell population was unaffected (Fig. 3.21 B and Fig. 3.22B). Conversely, the percent viability increased by ~14% with DOX-treatment in HES2 FL12 (Fig. 3.20 C). This was concurrent with a ~19% decrease in the apoptotic population, and a ~5% increase in the dead cell population in HES FL12 cells (Fig. 3.21 C and Fig. 3.22 C).

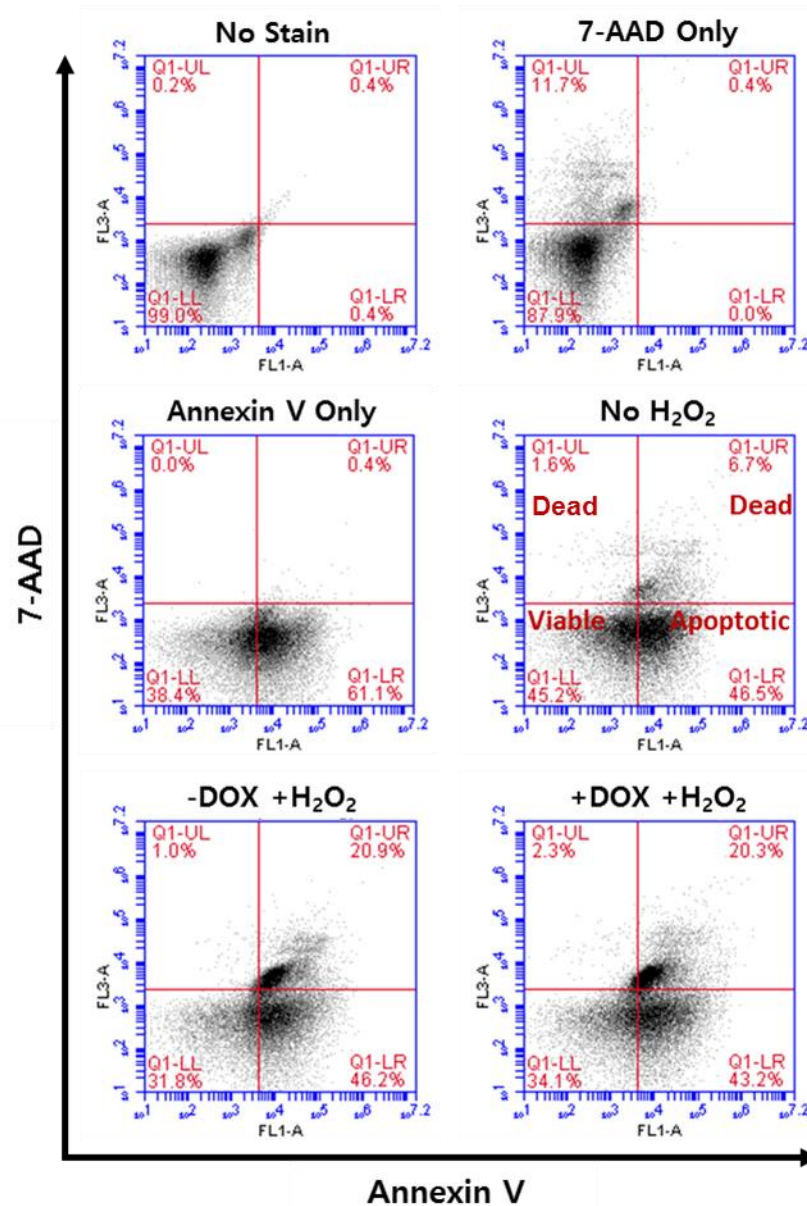


Fig. 3.19: Representative plots showing stain controls and gating strategies for flowcytometry experiment with 7-AAD and Annexin V for measuring cell death upon H₂O₂ treatment. 3xFLAG-hTERT isoform expression was induced via 1 μ g/ml DOX (2 days). Cells were grown under 2% O₂ and 100 μ M H₂O₂ was added the growth medium 2-hour prior harvest. Cells were stained with annexin V-FITC and 7-AAD to label apoptotic and dead cells, respectively. Measured cell population was first gated to exclude cell debris and background signals. Q1-UR: +Annexin V/+7-AAD (dead), Q1-UL: -Annexin V/+7-AAD(dead), Q1-LR: +Annexin V/-7-AAD (apoptotic), Q1-LL: -Annexin V/-7-AAD (viable).

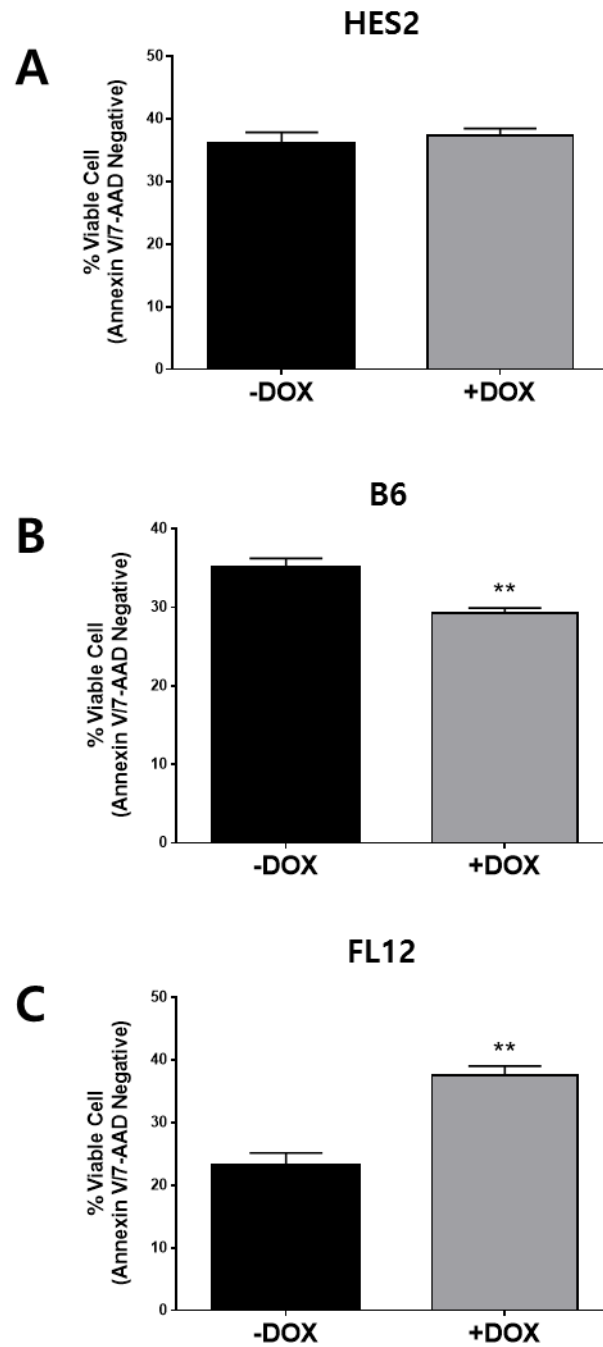


Fig. 3.20: Effect of DOX-induced 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT on cell viability in HES2 under H₂O₂-induced cell stress. Percent viable cell (Annexin V-FITC/7-AAD negative) plots in **A**) HES2, **B**) HES2 B6 **C**) HES2 FL12 (n=4, error bars = \pm SEM).

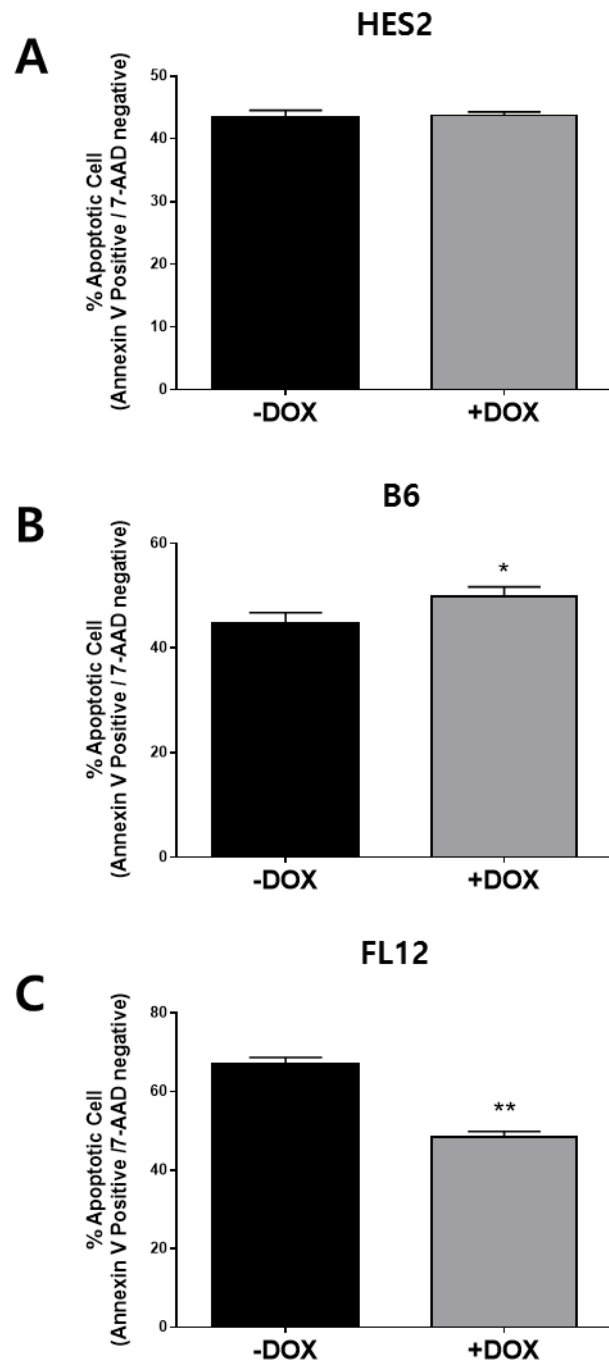


Fig. 3.21: Effect of DOX-induced 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT on apoptosis in HES2 under H_2O_2 -induced cell stress. Percent apoptotic cell (Annexin V-FITC positive/7-AAD negative) plots in **A) HES2**, **B) HES2 B6** **C) HES2 FL12** (n=4, error bars = \pm SEM).

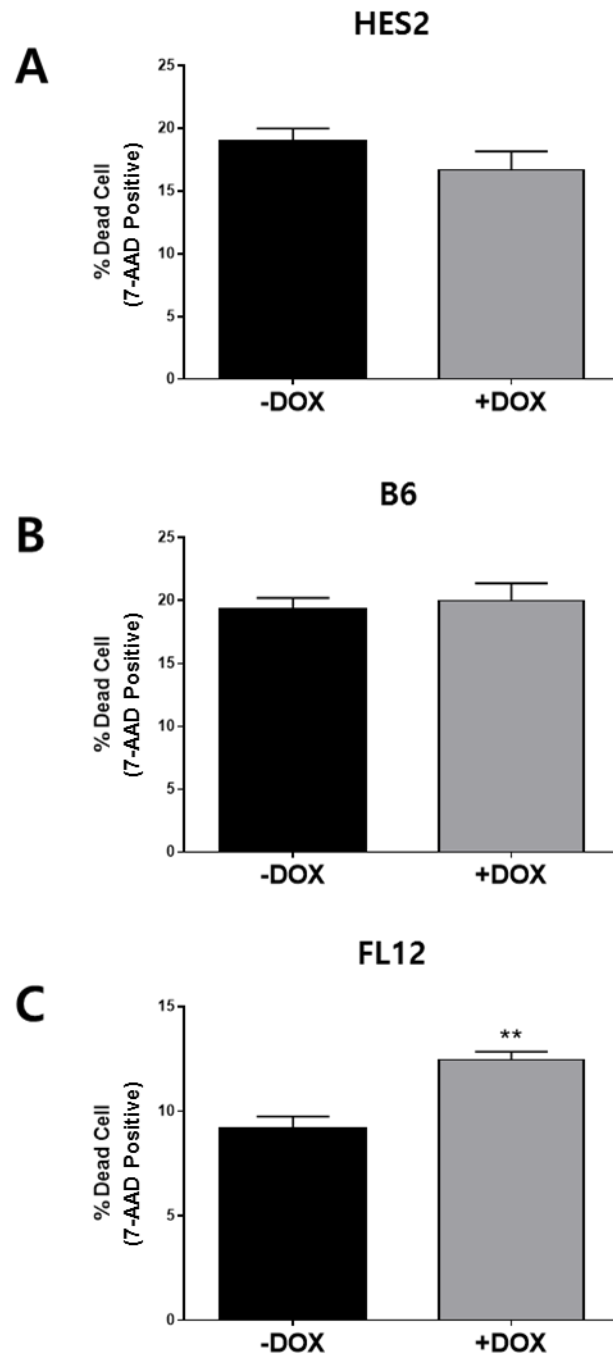


Fig. 3.22: Effect of DOX-induced 3xFLAG- β -hTERT and 3xFLAG-Full-Length-hTERT on cell death in HES2 under H₂O₂-induced cell stress. Percent dead cell (Annexin V-FITC/7-AAD positive) plots in A) HES2, B) HES2 B6 C) HES2 FL12 (n=4, error bars = \pm SEM).

Chapter 4: Discussion

The telomerase RNP complex plays a crucial role in maintaining telomere homeostasis. To enable rapid and indefinite cellular proliferation, cancer cell utilizes telomere-lengthening cellular machinery such as alternative lengthening of telomere (ALT) or telomerase. Several studies in human cancer and fibroblast cells have pointed to extra-nuclear translocation of telomerase catalytic subunit hTERT and its significance in extra-telomeric functions in modulating cellular behavior. Notably, alternative splicing of the TERT transcript to β -hTERT and its extra-nuclear localization have been previously reported in a subset of cancer cells. hESCs and cancer cells share similar cell physiology including high proliferative capacity, glycolysis-dependent energy metabolism, and high telomerase expression. In light of recent discoveries of hTERT's mitochondrial roles, I hypothesized that extra-nuclear translocation of FL-hTERT and β -hTERT will occur in hESC, and that they will collect at mitochondria under hydrogen peroxide-induced oxidative stress, thereby modulating mitochondrial functions. Owing to the lack of hTERT-specific antibody and the intricacy of hTERT alternative splicing, hTERT-overexpressing cell lines (doxycycline-inducible) were established by PiggyBac genomic integration of Flag-tagged (N-terminal) FL-hTERT and Flag-tagged β -hTERT mRNA sequences (*see* section 2.1.3). Various effects of doxycycline-induced hTERT expression were assessed in the clonal hESCs while paying extra-attention to the potential side effects of doxycycline and the Flag-fusion at the N-terminal of the TERT transgene.

4.1 Pluripotency and alternative splicing of endogenous hTERT in hESC

Prior to the overexpression studies, I began by verifying the pluripotent state of the hESC line HES2, cultured under 21% oxygen, by immunofluorescence microscopy. HES2

cells were marked immuno-positive for OCT4 and SSEA-4, and negative for SSEA-1 (Fig. 3.1 C) supporting their pluripotent state.

TaqMan RT-qPCR was used to detect endogenous hTERT transcripts isoforms containing neither α - nor β -deletion ($\alpha+\beta$ +hTERT), β -deletion (β -hTERT), α -deletion (α -hTERT), and both α - and β -deletion (α - β -hTERT). Importantly, each of the four primer sets ($\alpha+\beta$ +, β -, α -, α -) used for the PCR detects the presence of only the respective deletion, but does not distinguish between any other insertions/deletions that may be present on the same target transcript. For example, $\alpha+\beta$ +hTERT, as detected by TaqMan primers and probe, includes not only the full-length-(FL-)hTERT transcript but also $\alpha+\beta$ + transcript species with other deletions/insertions present elsewhere. To increase the specificity of detection, one could employ long-range PCR to amplify the whole lengths of hTERT transcripts.

My PCR results revealed that HES2 was indeed expressing certain hTERT isoforms exhibiting different combinations of α - and β -deletion when cultured under 21% oxygen. The transcript abundance of β -hTERT was proportionately the highest among $\alpha+\beta$ +, β -, α -, α - β -hTERT transcripts, and was as high as that of $\alpha+\beta$ +hTERT (Fig. 3.1 B). In a previous study by Ulaner *et al.* [78, 102], it was demonstrated that the splicing pattern of hTERT was tissue-, and developmental-stage-specific. In human embryonic kidney tissue, β -splicing occurs at the expense of FL-hTERT transcript, and this was proposed to be a mechanism involved in downregulation of telomerase activity. Similarly, Yan and colleagues [111] demonstrated a negative correlation of telomerase activity with the constituent ratio of deletion hTERT variants, especially β - hTERT. In hESCs, telomere length homeostasis is strictly maintained as excessively long telomeres can cause telomere instability and replication stress [196]. Therefore, it is highly possible that a similar regulation of telomerase activity by alternative splicing may be reinforced in hESC to prevent excessive lengthening

of telomeres. Notably, β -hTERT transcript persisted in human fetal kidney development even in the absence of all other hTERT isoforms (including the FL-hTERT), raising the possibility of β -hTERT's involvement outside canonical telomerase function.

I then examined how the splicing of endogenous hTERT changed when cells were cultured with 2% oxygen and different combinations of doxycycline and H₂O₂ (Fig. 3.2). When cells were cultured under 2% oxygen, the transcript composition of α + β -hTERT and β -hTERT remained nearly equal in quantity and higher than that of α -hTERT and α - β -hTERT compared to when the cells were cultured in 21% oxygen (Fig. 3.2 A and E). In contrast, our lab's previous work in H9 hESC line showed that α + β -hTERT and α -hTERT transcripts dramatically decreased going from 21% O₂ to 2% O₂ culture condition [184]. Radan *et al.* proposed that the change in splicing pattern might be attributed to the function of hypoxia-inducible-factor (HIF), based on its ability to regulate alternative splicing in hypoxic conditions [209 - 213]. The apparent discrepancy in our results may arise from cell line-specific differences and/or from the differences in our methodology. Although all hESC lines share the expression of characteristic pluripotency markers, they exhibit several differences owing to the wide range of factors such as variable culture conditions and the inherent genetic variation of the embryos from which embryonic stem cells were derived [199]. Thus, it is possible that the two cell lines used in this study (HES2) and by Radan *et al.* (H9) respond differently to the changing oxygen tension. Secondly, the outcome of the result may have greatly depended on the oxygen tension the cells were exposed to during maintenance prior to the hypoxic/normoxia treatment. Lastly, the duration of hypoxia and normoxia treatment varied between the two studies (5 days in the study by Radan *et al.* and 3 days for the current thesis). It would be interesting to see whether the alternative splicing patterns converge when the experimental differences are removed.

The splicing pattern changed when H₂O₂ or doxycycline was singly added to the culture media (Fig. 3.2 B and C). After 2-hour treatment of 100 μM H₂O₂, the percent transcript composition of β-hTERT was significantly higher than that of α+β+hTERT (Fig. 3.2 B). It appeared that the increased β-splicing came at the expense of α+β-splicing. However, no statistically significant change was seen with any of the four hTERT isoforms between control group (2% oxygen) and H₂O₂-treated group. A small sample size (n=3) may be preventing the observation of a statistically true event. Nonetheless, it appears evident that H₂O₂ alters the splicing pattern of hTERT in favor of β-hTERT. Other studies have shown a similar effect of H₂O₂ on alternative splicing of different genes through oxidative stress [212, 213] and mitochondrial damage [202]. Gilbert *et al.* [201] demonstrated that H₂O₂ directly affects the protein abundance of spliceosome complex proteins (hnRNPs, PTBP1), which suggests H₂O₂ can potentially alter the transcript splicing of many genes and, perhaps, hTERT. H₂O₂ is a cell membrane-permeable molecule that is naturally generated as byproduct of aerobic respiration [203], and can act as inter- and intra-cellular signaling molecule [204]. In a pathological context, H₂O₂ concentration greater than 50μM is generally considered cytotoxic, but this is largely cell-type and culture media-dependent as different cells possess differential resistance to oxidative stress [205, 206]. In mESC, 24 hour 150μM H₂O₂ treatment results in apoptosis, whereas shorter treatment time (2 hours, same concentration) resulted in transient cell cycle arrest [207]. Research conducted by Kumar *et al.* [208] demonstrated that treating hESC with 200 μM H₂O₂ for 30 minutes resulted in a significant increase in DNA damage. The concentration of H₂O₂ used in my study, 100μM, albeit lower than 200μM, was applied for much longer duration (two hours) than 30 minutes, and may have caused significant amount of DNA damage in the cells, triggering cell stress- and apoptotic signaling cascades. H₂O₂, in fact, is a poorly reactive molecule and undergoes

Fenton-reaction with intra-cellular iron (Fe^{2+}) to become the deleterious hydroxyl radical [209]. Intriguingly, Santos et al. [172] showed that ectopically expressed FL-hTERT in normal human fibroblasts cells promotes H_2O_2 -induced mitochondrial DNA damage by increasing bioavailability of divalent iron pool. Therefore, it is possible that the observed shift towards β -splicing may have been a rescue attempt to lessen the damage-amplifying effect of FL-hTERT.

Similarly, 2-day treatment of $1\mu\text{g/ml}$ doxycycline resulted in higher percent composition of β -hTERT transcript relative to that of $\alpha+\beta$ -hTERT (Fig. 3.2 C). This result indicates that doxycycline treatment has a side-effect on the splicing pattern of hTERT (and possibly other pre-mRNA transcripts) in hESCs. Whether doxycycline acts directly or indirectly on the splicing machinery of hTERT is unclear at this stage. Doxycycline is categorized as a tetracycline family antibiotic that is used to cure a broad spectrum of bacterial infections [209 – 211]. The antibacterial activity by tetracyclines is mediated by inhibiting the bacterial ribosome, effectively blocking bacterial protein synthesis [210]. Despite its frequent use in inducible mammalian transgene systems, significant evidence exists to show that tetracyclines can impair mitochondrial function in eukaryotic cells through the inhibition of mitochondrial ribosome, which may be explained by the stark similarity of bacterial ribosomes and eukaryotic mitochondrial ribosomes (in support of endosymbiotic bacterial origin of mitochondria) [212 - 215]. The effect of doxycycline extends to hESC and iPSC. Work by Chang *et al.* [216] showed that doxycycline improves viability and self-renewal of hESCs and iPSCs through activation of PI3K/AKT signaling, an important pathway for self-renewal of PSCs (*see* section 1.1.1). However, it is not known whether the activation of PI3K/AKT signaling is a result of mitochondrial inhibition by doxycycline. The PI3K/AKT signaling pathway controls numerous cellular processes such as

glucose metabolism, protein synthesis, and RNA processing. AKT can influence splicing regulation indirectly by phosphorylating serine/threonine-protein kinase 2 (SRPK2) and serine/threonine-protein kinase 1 (SRPK1; auto-phosphorylation), which in turn activates SR family proteins [217, 218]. Alternatively, AKT can directly phosphorylate SRSF1, SRSF7 and SRSF5 [219-221]. Considering β -deletion is also mediated by a SRSF family protein (SRSF11; *see* section 1.2.2), it is likely the case that doxycycline treatment acts on the splicing machinery of hTERT by first activating the PI3K/AKT signaling pathway and subsequently SRSF proteins. In addition, the fact that hTERT splicing pattern changed in favor of β -hTERT following the speculated activation of PI3K/AKT hints at a novel extra-telomeric role of β -hTERT in survival/self-renewal of hESCs and supports the currently existing evidence for hTERT regulation by alternative splicing. Additionally, DOX-treated samples showed reduced splicing of α - β -hTERT in comparison to non-doxycycline treated groups (Fig. 3.2 E 4th and 5th columns). This relative reduction of α - β -hTERT transcript population may account for the shift of splicing toward β -hTERT transcript following doxycycline treatment.

When doxycycline and hydrogen peroxide treatments were combined, strikingly, the proportion of β -hTERT transcript dropped down to the level of α + β +hTERT, while the α - β -hTERT transcript remained low as seen in doxycycline-treatment only group. It is tempting to look for an explanation for this phenomenon through mitochondria since they are the known off-targets of doxycycline and are the central hub of intra-cellular free iron sequestration for the downstream heme and iron sulfur syntheses [221, 222]. In this context, the side effect of 2-day long doxycycline treatment (impairment of mitochondria) might have had a serendipitous effect of curtailing the free bivalent iron pool by the time H₂O₂ was introduced to the cells, reducing the generation of highly deleterious hydroxyl radicals.

Assuming the previously seen increase in β -hTERT splicing played a protective role (when doxycycline and hydrogen peroxide were applied singly), one may speculate that the cytotoxic effect of H_2O_2 might have been alleviated by mitochondrial impairment and AKT activation by doxycycline, leading to the overall reduction of intra-cellular stress and the resultant decrease in β -splicing. Future experiments that examine changes in intra-cellular ROS and AKT activation after DOX- and H_2O_2 treatments will be necessary to better explain this multi-faceted result. α - β -hTERT, despite the reduced β -splicing, remained unchanged, which suggest an independent role for α - β -hTERT isoform may exist.

4.2 Transgenic HES2 line B6 and FL12 remain pluripotent following doxycycline and H_2O_2 treatment.

For delineating TERT isoform function and to enable reliable detection of hTERT transgenic proteins, multiple transgenic HES2 lines were clonally established for DOX-inducible overexpression of 3xFLAG-tagged β -hTERT and FL-hTERT transgenes. Four clonal cell lines established from HES2 β -hTERT (B2, B3, B6, B7) and HES2 FL-hTERT (FL7, FL9, FL10, FL12) were randomly chosen and analyzed for FLAG-tagged protein expression (appendix A3: Fig. 3.1). Based on preliminary results of 3xFLAG-hTERT transgene induction 12-hours post doxycycline, clonal cell line selection was further narrowed down to the least expressive β -clone HES2 B3, the most expressive HES2 B6, a FL-clone (FL9) with similar level of protein induction as B6 HES2, and the most expressive FL-clone HES2 FL12. The level of transgene induction in the selected clones was re-examined by RT-qPCR using transgene-specific primers (n=1; I focused more on the protein because the goal of my study relies on the protein stability/expression rather than the

transcript stability) and by Western blot (Fig. 3.3). In all clones, both 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT expression peaked after 6 hour of doxycycline treatment and remained relatively unchanged over the 3 day-long doxycycline time course.

Interestingly, the steady-state protein level of 3xFLAG- β -hTERT was significantly lower (approximately by 7-fold) than that of 3xFLAG-FL-hTERT (Fig. 3.3 C) regardless of the duration of doxycycline. This can arise from multiple factors including transcriptional activity at the promoter site of the recombinant gene, the stability of 3xFLAG- β -hTERT transcript, and protein degradation.

The low protein expression of 3xFLAG- β -hTERT may result from diminished transcription of the transgene. 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT expression cassettes utilize identical promoter (pCAG), transactivator (TA), activator (DOX), and transactivator responsive element (TRE) (Fig. 2.1). Therefore, the differences in transcriptional activity of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT would depend on the site of and the multiplicity of transgene integration.

In my preliminary characterization of transgenic transcript level by RT-qPCR (Fig. 3.3 A, n = 1), I observed that 3xFLAG- β -hTERT transcripts slowly decrease over 3 days of doxycycline treatment in both HES2 B3 and B6, despite daily renewal of doxycycline in the growth media. Previously, it was shown that the NMD activity on β -hTERT transcript can vary in different cell types [102]. Greater than 90% depletion of UPF1 (core component of NMD machinery) resulted in 10-fold increase in β -hTERT transcript in UM-UC-3 cell line (bladder cancer), an indication that β -hTERT transcript can be targeted by NMD. Conversely, β -hTERT transcript in BT-549 cells (breast cancer; normally expresses high level of β -hTERT) did not change significantly with the knockdown of UPF1, suggesting β -hTERT

transcript can escape NMD pathway depending on the cell type. Therefore, it would be interesting to perform a similar experiment to test the NMD sensitivity of β -hTERT transcript in hESC.

The steady-state protein abundance of 3xFLAG- β -hTERT (B6) was consistently ~5-fold less than that of 3xFLAG-FL-hTERT (FL12) at all doxycycline treatment times (Fig. 3.3 C), whereas 3xFLAG- β -hTERT is only ~2-fold less than 3xFLAG-FL-hTERT at transcript level (Fig. 3.3 A). Adding to this, the steady-state transcript of 3xFLAG-FL-hTERT in FL9 clone was ~3 times less than 3xFLAG- β -hTERT (B6), yet the protein abundance was ~3 times higher at all doxycycline durations (Figure. 3.3 A and C). Listerman *et al.* and our lab previously confirmed the translational competency of β -hTERT transcript [100, 186] and α - β -hTERT transcript [184] by polysome profiling. Collectively, these findings point to the possibility that 3xFLAG- β -hTERT protein has higher propensity to degrade than 3xFLAG-FL-hTERT. FL-hTERT is known to degrade by ubiquitination pathway [131– 133], but little is known about the protein degradation of β -hTERT. β -hTERT lacks a large portion of the C-terminal end (Fig. 1.1 B), which is necessary for MKRN1-mediated ubiquitination/ degradation of hTERT[129]. Therefore, if 3xFLAG- β -hTERT is indeed degrading via ubiquitination, CHIP [130] or Hdm2 [131] may be at the focus of such regulation (*see* section 1.2.4).

I decided to work with the most expressive clones HES2 B6 and HES2 FL12 for the robustness of potential downstream effects and to adequately compare functional differences between overexpression of the two TERT isoform transgenes in hESCs. For the treatment time of doxycycline, I settled with 2 days of transgene induction by doxycycline for both B6 and FL12 clones for maximum transgene expression (Fig. 3.3 A).

In the light of discoveries highlighting the importance of hTERT [185] and telomere maintenance [224-228], I performed immunofluorescence microscopy to assess the effect of TERT isoform transgene expression on the pluripotent status of the selected clones. Both HES2 B6 (Fig. 3.4) and HES2 FL12 (Fig. 3.5) stained positively for OCT4/SSEA-4, and negatively for SSEA-1, indicating their pluripotent state under normal culture conditions (2% oxygen, no H₂O₂ or doxycycline added). Following doxycycline (1 μg/ml for 2 days) and/or H₂O₂ treatment (100 μM for 4 hours), the cells stained in the same pattern and apparent intensity, suggesting, at least at the visible level, that pluripotency was not altered by the cell treatments (Fig. 3.4 and Fig. 3.5). This supports a previous study in mESC which showed that short-term treatment of H₂O₂ (150 μM for 2 hours) did not affect the expression of pluripotency marker genes Oct4, Sox2, and Nanog [207]. However, no quantification was performed on the immunofluorescence intensities so it is possible that the pluripotency markers were changing over the course of cell treatments. Many cells were non-adherent and round in morphology (data not shown) by the end of 4-hour H₂O₂ treatment, which suggests the cells were responding to the acute oxidative stress by switching on the apoptotic pathway rather than by differentiating (loss of pluripotency). In support of this, Guo *et al.* demonstrated that mESCs, despite their increased resistance to senescence by oxidative stress, undergo apoptosis when treated with H₂O₂ at dose higher than 150 μM for 24 hours [207]. In the same study, a significant portion of mESCs treated with 150 μM H₂O₂ for 3 hours lost their ability to adhere to the surface of culture dish, and failed to recover when resuspended in fresh culture medium. This closely resembles my observation with H₂O₂-treated hESC, and likely signifies a pre-apoptotic event that ultimately contributes to H₂O₂-induced cell cycle arrest and apoptosis.

4.3 3xFLAG-hTERT in HES2 B6 and HES2 FL12 bind TERC and RMRP.

To show the competency of the 3xFLAG-tagged-hTERT species in carrying out the known functions of non-tagged versions of hTERT, I tested their RNA-binding ability (Fig. 3.6 and 3.7) and telomerase activity levels (Fig. 3.8) of the FLAG-tagged hTERT isoforms in HES2 hESCs. In FLAG-RIP experiments for TERC-binding, the amount of TERC recovered was significantly higher when doxycycline was added for both HES2 B6 and HES2 FL12 (Fig. 3.7 B and C, respectively), but not for parental HES2 cells. The insignificant change observed with parental HES2 demonstrates that the significant differences observed in HES2 B6 and HES2 FL12 were not due to a side-effect of doxycycline and/or because of nonspecific binding of TERC to the FLAG-antibody-conjugated beads. Together, the experiment confirms that both 3xFlag-tagged β -hTERT and FL-hTERT transgenic proteins can bind TERC *in vitro*. In a side-by-side comparison (Fig. 3.7 A), the fold-recovery of TERC was significantly higher for 3xFLAG-FL-hTERT (~60-fold increase) than for 3xFLAG- β -hTERT (~10-fold increase). Interestingly, a similar RIP experiment by Listerman et al. [102] confirmed Flag-tagged β -hTERT bound to TERC. In stark resemblance with my results, Flag-tagged FL-hTERT co-immunoprecipitated only a 3-fold higher amount of TERC than Flag-tagged β -hTERT. It is tempting to conclude that FL-hTERT has greater affinity for TERC than β -hTERT. However, making any conclusions on the affinity would be inappropriate for a few reasons. First, my data for DOX-induced recombinant protein expression showed that the steady-state abundance of 3xFLAG-FL-hTERT was significantly higher than that of 3xFLAG- β -hTERT by approximately 5-fold (Fig. 3.3, *see* section 4.2). This disparity between the two cell lines can also lead to similar reduction in TERC-recovery by 3xFLAG- β -hTERT. Second, there is no way of knowing whether FLAG-hTERT proteins were bound to the immunoprecipitation beads to saturating capacity or not. In other words, if

the same volume (30 μ l packed volume) of anti-FLAG beads were binding 3xFLAG-FL-hTERT to saturation, but not 3xFLAG- β -hTERT (possibly because of significantly low steady-state protein abundance of 3xFLAG- β -hTERT; see the beginning part of this section), no proper comparison can be made between 3xFLAG-FL-hTERT and 3xFLAG- β -hTERT since the amount of immunoprecipitated 3xFLAG-hTERT species differs. Thirdly, the fact hTERT can form dimers adds another layer of complexity. As shown in Fig. 1.1, β -hTERT lacks one of two oligomerization domains. If 3xFLAG- β -hTERT is less likely to self-dimerize than 3xFLAG-FL-hTERT *in-vitro*, it is possible that the same amount of saturated anti-FLAG-beads were pulling down different amount of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT. Further investigation is required to clarify this.

RMRP is another RNA partner of hTERT. The domain for RMRP-binding on the protein is currently unknown [181]. I performed the identical RIP experiment as above to estimate the recombinant hTERTs' ability to bind RMRP. As expected, no difference was seen amongst HES2 control group samples (Fig. 3.7 D). Similarly, RMRP recovery in HES B6 hESCs treated with doxycycline was not statistically different from its non-DOX-treated counterpart. The inherently low expression level of 3xFLAG- β -hTERT of HES2 B6 clone may have contributed to the low RMRP yield in DOX-treated group (Fig. 3.3, *see* section 4.1). However, considering the identical doxycycline treatment (thus, presumably the same level of 3xFLAG- β -hTERT protein expression) used for TERC-RIP resulted in significant TERC recovery (Fig. 3.6 B), the low protein expression by HES2 B6 may not be the limiting factor. This points to the possibility that β -hTERT has impaired RMRP binding. Importantly, HES2 control group samples (Fig. 3.7D) and HES2 B6 samples (Fig. 3.7 B) precipitated comparable amount of RMRP, which suggests a substantial amount of RMRP might have been bound to the RIP beads non-specifically. The hypothesized non-specific precipitation

could have led to false positive reading of RMRP in non-DOX-treated samples, masking the truly immunoprecipitated (by 3xFLAG- β -hTERT) RMRP in DOX-treated HES2 B6. In support of a previous study by Maida *et al.* [164], I observed ~5-fold increase in RMRP recovery upon the addition of doxycycline to HES2 FL12, indicating the interaction of 3xFLAG-FL-hTERT with RMRP (Fig. 3.7 C). In a side by side comparison of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT (Fig. 3.7 A), 3xFLAG-FL-hTERT co-precipitated with approximately 2-fold more RMRP than 3xFLAG- β -hTERT. However, as discussed above, more optimization and redesigning of the experiment is necessary to compare binding affinities of RNA components between TERT isoforms.

4.4 The effect of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT on the telomerase activity in HES2 B6 and HES2 FL12.

The effect of overexpressing 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT on telomerase activity in hESCs was investigated (Fig. 3.8). hESCs express endogenous telomerase reverse transcriptase (TERT) and exhibit high telomerase activity [184]. β -hTERT can act as a dominant negative inhibitor of telomerase activity by sequestering TERC [102]. It was proposed by the same study that this mode of inhibition is effective only when TERC is the limiting factor for telomerase activity. Potential side-effects of doxycycline on endogenous telomerase activity in non-transgenic HES2 was first tested. I demonstrated that doxycycline had no effect on endogenous telomerase activity levels since no significant difference in the activity was observed with doxycycline treatment compared to non-treated controls hESCs (Fig. 3.8 B first bar plot).

As expected, overexpression of 3xFLAG- β -hTERT led to significantly reduced levels of telomerase activity (Fig. 3.8 B second bar plot). This inhibition could result from several reasons. As previously suggested, the available pool of free TERC transcripts may be in limiting supply in hESCs and reduced by binding to catalytically inactive 3xFLAG- β -hTERT, decreasing the number of active telomerase RNP complexes [102]. Although there is no confirmation on the formation of heterodimeric β -hTERT/FL-hTERT to date, such dimerization seems plausible given β -hTERT retains oligomerization domain for interaction with another hTERT protein [188]. Hence, one can speculate that the heterodimerization of 3xFLAG- β -hTERT with the endogenous FL-hTERT may have caused an inactivation of the complex, which could additionally account for the observed inhibition of telomerase activity.

Based on previous studies that showed TERC is in excess for telomerase complex formation in human immortalized cells [82, 226], I had anticipated that overexpressing 3xFLAG-FL-hTERT would lead to increased total telomerase activity. Surprisingly, the overexpression of 3xFLAG-FL-hTERT did not significantly increase telomerase activity levels (Fig. 3.8 B third bar plot). To seek an explanation for this unexpected outcome, I stepped back and examined what was happening to the total hTERT (endogenous and transgenic) population at the transcript level (Fig. 3.9) by TaqMan qPCR (Fig. 2.1 B). Upon adding doxycycline to HES2 FL12, not only did the transcript abundance of α + β -hTERT increased by ~500 fold, those containing β - transcript species (β - and α - β -hTERT) also increased greatly by ~300 fold (Fig. 3.9 A). The transgenic DNA sequence for 3xFLAG-FL-hTERT directly codes for the processed TERT mRNA species and does not include introns which are required for the alternatively splicing of β -hTERT [102]. This suggests that the observed increase in β -hTERT- and α - β -hTERT transcripts is due to the increased β -splicing of *endogenous* hTERT pre-mRNA. Moreover, doxycycline is unlikely a part of this as I had

seen no such tremendous increase in β -splicing in DOX-treated non-transgenic HES2 (Fig. 3.2 C and E). This is very interesting result which demonstrates the cell's ability to respond to ectopic expression of 3xFLAG-FL-hTERT. In other words, a feedback mechanism may exist in hESCs to sense the aberrant increase in $\alpha+\beta$ -hTERT transcript abundance and/or telomerase activity levels (canonical and/or non-canonical) of FL-hTERT and to downregulate it by switching to β -favoring splicing. In support of this, similar hTERT regulation by alternative splicing had been reported previously [79, 80, 110] (*see* section 1.2.2). Thus, the significant increase in non-catalytic hTERT transcripts (β -hTERT and α - β -hTERT) due to DOX-induced 3xFLAG-hTERT overexpression may be attributed to my failure to observe an increase in telomerase activity within this transgenic cell line. Notably, DOX-treatment in HES2 B6 only induced β -hTERT (~1000-fold induction) and α - β -hTERT (~200-fold induction). Since α -deletion is mediated by an alternative splice acceptor within exon 6 (therefore present in the 3xFLAG- β -hTERT transgene), The increase in α - β -hTERT transcript level may result from an additional α -deletion on the DOX-induced 3xFLAG- β -hTERT transcript.

The N-terminal end of hTERT is essential for telomerase activity as it contains an anchor site (TEN domain) to stabilize telomeric DNA-to-hTERT interaction [226], and TEN domain mutations are known to cause defective telomere maintenance *in vivo* [227]. Therefore, one may speculate that the N-terminal 3xFLAG could potentially disrupt this particular function of the TEN domain, leading to the failure of 3xFLAG-FL-hTERT to remain bound to the telomere-like DNA substrate in telomerase activity assay. However, several previous reports demonstrated unobstructed telomerase activity by N-terminal 3xFLAG-tagged hTERT, suggesting that N-terminal 3xFLAG-tagging does not affect the telomerase activity *in-vitro* [71, 231, 232].

4.5 The intra-cellular localization of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT in hESCs treated with H₂O₂-induced oxidative stress.

Several studies previously reported mitochondrial localization of FL-hTERT [187, 189, 203, 208, 214] and β -hTERT [149] under oxidative stress. We previously shown that hTERT was more extra-nuclear localized in hESCs cultured under 21% oxygen conditions compared to the prominently nuclear TERT observed in cells cultured in 2% oxygen [184]. Western blot of sub-cellular fractions and immunofluorescence microscopy were used in this study to determine the intra-cellular localization of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT when cells were treated with the characterized dose and duration of H₂O₂.

The amount of 3xFLAG- β -hTERT in mitochondrial and nuclear fractions did not change significantly over the time course of hydrogen peroxide treatment (Fig. 3.10 A and B). Similarly, no change in 3xFLAG-FL-hTERT was observed with H₂O₂ treatment in either fractions (Fig. 3.11 A and B). An independent set of the identical experiment was conducted with prohibitin as mitochondrial marker protein (for normalization of mitochondrial protein; appendix A4: Fig. 3.2). It showed similar results to when VDAC was used as the mitochondrial fractionation and loading control. Importantly, the fractionation quality (as determined by the presence of mitochondrial/nuclear markers in fractions) was rather inconsistent between experimental replicates (Fig. 3.10 and Fig. 3.11; data not shown), leading to highly variable results (Fig. 3.10, Fig. 3.11; appendix A4: Fig. 3.2; *see* error bars). hESCs exhibit high nuclear-to-cytoplasm ratio [230], and mitochondria of hESC are located in close proximity with the nuclei [230, 231]. Such intrinsic cell characteristics make clean separation of the two organelles very difficult [232– 234], a task I could not fully overcome.

As an alternative method to fractionation, immunofluorescence imaging was performed on fixed HES2 B6 and HES2 B12 cells after H₂O₂ treatment (Fig. 3.12). There was no apparent co-localization of either 3xFLAG-β-hTERT or 3xFLAG-FL-hTERT with mitochondria after H₂O₂ treatments. 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were predominantly nuclear with only minute amount of 3xFLAG-hTERT localized to the cytoplasmic space. Our lab previously observed extra-nuclear localization of endogenous hTERT in H9 line under chronic exposure to 21% O₂ for 5 days [184]. However, I failed to observe this in HES2 FL12 that had been exposed to 21% O₂ for 3 days (appendix A5: Fig. 3.3). there were some nuclei with 3xFLAG-hTERT located very close to the nuclear boundary (Fig 3.12 indicated by white arrow). I believed this intra-nuclear localization pattern might be the influence of H₂O₂-induced oxidative stress, and performed a blinded cell-count analysis on immunofluorescence microscopy images (Fig. 2.2 and Fig. 3.13). Unfortunately, the analysis revealed no significant effect of hydrogen peroxide on 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT localization within hESCs.

The observed localization pattern of 3xFLAG-FL-hTERT is particularly puzzling as it directly contradicts previous studies that demonstrated extra-nuclear-[184] and mitochondrial [185, 186, 197, 240] localization of FL-hTERT under H₂O₂-induced oxidative stress. These studies employed wide range of dose and duration of oxygen tension/hydrogen peroxide [186, 187, 197], or no oxidative stress at all [175]. The dose of hydrogen peroxide used in these studies vary from 200μM to 500μM, and the duration of H₂O₂ treatment ranged from 3 hours to 5 days. The different cell types used in the studies (NHF, HeLa [175], fetal human lung fibroblast cell MRC5 [195], breast cancer cell MCF7 [183]) add extra layer of complexity to this, making it nearly impossible to predict the right dose and time window of H₂O₂ to use in hESC for inducing mitochondrial localization of hTERT. In my preliminary

immunofluorescence experiments with earlier (15 minutes, 30 minutes, 1 hour) and longer duration (4 hours, at which point the cells were apoptotic; *see* last paragraph of section 4.1) of 100 μ M H₂O₂ treatment (data not shown), 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT remained mostly nuclear. Based on this, it is likely that I am targeting right (inducing cell stress) dose and sufficiently wide time window of H₂O₂ treatment. Considering the hTERT investigated in previous studies were either endogenously expressed protein [195] or tagged with relatively small tag such as myc (1.2 kDa) [186, 240], the large size of 3xFLAG tag (2.8 kDa) used in my study may be physically hindering the trans-membrane translocation of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT. However, EGFP (32.7 kDa)-tagged hTERT used by Santos et al.[175] was able to cross both nuclear and mitochondrial membrane, precluding this possibility. Importantly, the mitochondrial targeting sequence (MTS) of hTERT lies at the N-terminal end. Thus, the location (N-terminal vs C-terminal) and the amino acid (a.a.) constituents of the tagging can greatly influence the proper function of MTS. Mitochondrial targeting sequences, as outlined in a review by Walter Neupert [237], are generally 20-60 amino acid long, rich in positively charged a.a., and have the tendency to fold into amphiphilic α -helices. 3xFLAG is particularly enriched in negatively charged aspartate residues [238]. The negative charge on N-terminally tagged 3xFLAG may negate the positive charge on MTS, ultimately disabling MTS and abolishing mitochondrial localization. However, this does not fully explain my failure to observe not only the mitochondrial translocation but also the extra-nuclear localization of 3xFLAG-hTERT.

Phosphorylation by Src at tyrosine 707 is required for nuclear export of hTERT by CRM1/Ran interaction near the C-terminal of hTERT [142] (Fig 1.2 B). β -hTERT does not have this C-terminal region due to PTC-mediated truncation, and it is possible that 3xFLAG- β -hTERT was trapped inside nuclei because of the impaired CRM1/Ran-mediated nuclear

export. Under the assumption that the two ends of the protein come into close proximity in the native state, the N-terminal 3xFLAG-tag may block CRM1-hTERT interaction through steric hindrance, leading to nuclear trapping of 3xFLAG-FL-hTERT. For clarification, it would be necessary to investigate whether Y707 phosphorylation of FLAG-tagged TERT isoforms occurs upon H₂O₂ treatment in hESCs.

4.6 Investigation of the effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on mitochondria function in hESCs.

Ahmed *et al.* [195] demonstrated that ectopic expression of FL-hTERT in human fetal lung fibroblast cells results in increased mitochondria membrane potential and decreased mitochondria DNA content. Of note, the study did not consider whether mitochondrial localization of FL-hTERT was required for such changes. In my study, the potential mitochondrial changes in hESC after overexpressing 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were assessed.

Mitochondria membrane potential (MMP) was estimated by FL2 (fluorescence signal from J-aggregate, indicator of high membrane potential) to FL1 (fluorescence signal from JC-1 monomer, indicator of low membrane potential) ratio using flow cytometry (see section 2.5.1; Fig. 3.14 and 3.15). To account for the possibility of doxycycline side effect, DOX-treated non-transgenic HES2 was include in the experiment. The fold change in mitochondrial membrane potential (MMP) with DOX-treated HES2 F12 and HES2 B6 did not differ from DOX-treated non-transgenic HES2 control (Fig. 3.15). My failure to observe increase in MMP in DOX-treated HES2 FL12 may owe to the fact that DOX-induction in this cell line increases not only the 3xFLAG-FL-hTERT expression but also other hTERT

isoforms containing β -deletion (*see* section 4.2 of discussion). Certain mitochondrial roles of hTERT, such as DNA damage-amplifying [235] and mitochondrial ROS-reducing effects [173] requires the catalytically active form of hTERT. To date, there is no concrete evidence indicating that MMP-enhancing role of FL-hTERT strictly requires its presence in mitochondria, a large body of MMP-determining factors are nuclearly transcribed [65, 243]. control In this context, sufficiently expressed β -hTERT, a catalytically inactive form, could mask, and even inhibit the MMP-enhancing role of FL-hTERT from nuclei (recall 3xFLAG- β -hTERT remained in nuclei; *see* section 4.5). The fold change in MMP of DOX-treated HES2 B6 decreased slightly in comparison to that of HES2 control, but was not statistically significant ($p = 0.0635$, paired t-test). Considering the low level of DOX-induction of 3xFLAG- β -hTERT in HES2 B6 (see section 4.2), I believed that a significant change in MMP may be observed if 3xFLAG- β -hTERT can be overexpressed more robustly.

The effect of overexpressed 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT on mitochondrial DNA (mtDNA) copy number was examined by qPCR. I confirm a side-effect of doxycycline by showing that mtDNA content decreases by ~20% in non-transgenic HES2 after doxycycline treatment. (Fig. 3.16 A). As previously mentioned, several past studies have shown that tetracycline family drugs can impair eukaryotic mitochondrial function by inhibiting mitochondrial ribosome [212 - 215], which suggests doxycycline may be hindering the mtDNA synthesis process. Interestingly, the reduction in mtDNA content was not detected when 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT were overexpressed with doxycycline (Fig. 3.16 B and C). It was proposed that mtDNA synthesis involves priming of a nascent RNA from light strand promoter (LSP) [240] by a RNase formed between mitochondrial RNA processing endoribonuclease (RNase MRP) and RMRP (RNA component of the mitochondrial RNA-processing endoribonuclease) [241]. Studies by Maida

et al. [178, 185] and my earlier results (Fig. 3.7) demonstrated the interaction of RMRP with FL-hTERT and β -hTERT. In the context of successful mitochondrial localization of RMRP-bound 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT, the available pool of RMRP in mitochondria may increase, promoting mtDNA synthesis. However, my previous results indicate no mitochondrial localization of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT in hESCs. Therefore, an intricate interplay between hTERT and RNase MRP (also known to be both nuclear and mitochondrial [242]) may exist to regulate mitochondria DNA synthesis from the nucleus.

To further investigate the regulation of mitochondria by nuclear 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT, the expression of genes involved in mitochondria biogenesis (Fig. 3.17) and function (Fig. 3.18) was measured by RT-qPCR. PGC1 family proteins (PGC1A, PGC1B, PRC) are well known regulators of mitochondria biogenesis [243]. PGC1B, together with NRF1 (nuclear respiratory factor 1), stimulates the expression of target genes including regulators of mitochondria DNA replication (TFAM and POLG) [244] and mitochondrial transcription (TFAM and TFB1M) [240]. In support of the results from my mtDNA copy number experiment, the overexpression of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT did not affect the transcript abundance of any of the mitochondria biogenesis regulators (Fig. 3.17). Moreover, doxycycline treatment did not change the transcript abundance in HES2 (non-transgenic hESCs) (Fig. 3.17). This result indicates that DOX-induced reduction in mtDNA copy number observed earlier (Fig. 3.16) is not mediated by changes in nuclear transcribed genes, but more likely through direct intervention of doxycycline with the mitochondria. In terms of function, mitochondria of hPSC contain high levels of Mn-SOD2 (antioxidant enzyme)[66] and UCP2 (uncoupler protein)[245]. The steady-state transcript level of these genes also did not change with doxycycline treatment in any of the HES2 cell

lines (Fig. 3.18). Notably, UCP2 transcript level in all HES2 cells lines was much higher than other genes measured simultaneously, which supports the notion that HES2 is pluripotent cell with metabolic reliance on glycolysis (through down-regulation of OXPHOS through removal of carbon intermediates in the mitochondria matrix) [246].

4.7 The effect of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT on cell survival of HES2 lines under H₂O₂-induced oxidative stress.

H₂O₂ exposure at a high concentration can lead to generation of highly deleterious hydroxyl radicals and damage various cell components including lipids, proteins, and DNA. I conducted flow-cytometry experiments to measure cell survival/death in the various HES2 hESC lines under H₂O₂-induced oxidative stress.

First, the potential side effect of doxycycline on cell viability (Annexin V-FITC⁻/7-AAD⁻), apoptosis (Annexin V-FITC⁺/7-AAD⁻), and cell death (7-AAD⁺) was assessed by flow cytometry in nontransgenic HES2 cells (Fig. 3.20-22 A). Our results show that doxycycline has no effect on cell survival under H₂O₂-induced cell stress (Fig. 3.20-22 A).

When 3xFLAG- β -hTERT overexpression was induced via doxycycline, % cell viability decreased (Fig. 3.20 B) while the apoptotic population increased in HES2 B6 (Fig. 3.21 B). However, the dead cell population remained unchanged (Fig. 3.22 B). On the other hand, when 3xFLAG-FL-hTERT was overexpressed via DOX, the viable population dramatically increased (Fig. 3.20 C) while the apoptotic population decreased (Fig. 3.21 C). In cancer research, there have been several reports demonstrating anti-apoptotic role of hTERT against chemotherapeutic drugs [251-254]. In research conducted by Cao *et al.* [248], a short-term knock down of endogenous hTERT (measurements were taken 24 hours post

antisense RNA-mediated knock down) in breast cancer cells resulted in increased apoptosis without change in telomere length. The hTERT-depletion-mediated apoptosis was overcome by overexpression of catalytically active *and* inactive hTERT, suggesting anti-apoptotic/extra-telomeric role of hTERT and its non-catalytic versions. In my study, however, the cell survival of H₂O₂-treated hESC is enhanced by 3xFLAG-FL-hTERT, not by 3xFLAG-β-hTERT. It is highly possible that 3xFLAG-β-hTERT and hTERT mutant used by Cao *et al.* behave differently due to their structural differences. Moreover, the types of cells and stress used in the studies are completely different, which can also contribute to the disparate outcome. The pro-survival role of hTERT is mediated through modulation of Bcl2/Bax and the resultant mitochondria-dependent apoptosis pathway[251, 253].

Importantly, the inhibition of Bax-dependent apoptosis signaling was simultaneous with mitochondrial localization of hTERT. Therefore, my failure to observe mitochondrial localization of 3xFLAG-hTERT suggests that the augmented cell viability following doxycycline treatment in HES2 FL12 is possibly independent of Bax pathway. There has been several reports of the protective role of hTERT on mitochondrial [188, 225] and nuclear[183] DNA. All three studies proposed that DNA-protection by hTERT is mediated through reduction of mitochondrial generated ROS. hESCs have minimally active OXPHOS activity, and have lower mitochondrial ROS than its differentiated progeny [66]. Therefore, it is likely that the cells were exposed to an exogenous source of ROS (H₂O₂) that is far more damaging than the endogenous ROS generated by mitochondria of hESC. It is speculated that the anti- and pro-survival effect exhibited by 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT, respectively, would result from fundamental changes in anti-oxidant/DNA-repair mechanisms.

In addition, we show that DOX-induction in FL12 slightly increased dead cell population by ~2.5% (Fig. 3.22 C). At first glance, this may be puzzling because we demonstrated in the same experiment that DOX treatment in FL12 led to increase viability and decreased apoptosis. However, knowing that DOX-treated FL12 accompanies a significant increase in β -hTERT and α - β -hTERT transcript (Fig. 3.9 A), one can speculate that the molecular interactions between DOX-induced 3xFLAG-FL-hTERT and endogenous β -hTERT isoforms may lead to increased cell death.

Considering 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT are predominantly nuclear under the treatment conditions (Fig. 3.12), I believe that the effect of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT on cell survivability under oxidative stress is likely mediated through its nuclear regulation, not through mitochondria. NF- κ B is a key regulator of pluripotent state of hESC (*see* 1.3.2) and executes diverse cell functions including survival and apoptosis. Importantly, the nuclear translocation of p65 (one of five transcription factors in NF- κ B signaling) is facilitated by its direct molecular interaction with hTERT and *vice versa* [255, 256]. In the light of recent discovery demonstrating H₂O₂-activated p65 and its nuclear translocation [253], it is highly likely that NF- κ B plays a major role in the nuclear localization/retention of 3xFLAG- β -hTERT and 3xFLAG-FL-hTERT following H₂O₂ treatment, and their transcriptional activity in the nucleus to modulate cell survival under oxidative stress. For a future experiment, the molecular interaction between p65 and 3xFLAG- β -hTERT/ 3xFLAG-FL-hTERT should be tested using anti-FLAG-immunoprecipitation.

4.8 Concluding remarks and proposed future studies

hTERT remains a difficult protein to study due to its vastly diverse functions in different organelles, and for the poorly characterized mechanisms of its alternative splicing and isoforms. In this thesis, I show that hTERT transcript is alternatively spliced into isoforms carrying both α -deletion and β -deletion (α - β -), an α -deletion (α -), a β -deletion (β -), and neither deletion (α + β +) in hESC. The alternative splicing of hTERT pre-mRNA was affected significantly by both doxycycline and H₂O₂ treatments. It would be worthwhile to examine the physiological significance of the altered splicing in a future study. Using 3xFLAG-tagged recombinant hTERT proteins, I showed that 3xFLAG- β -hTERT overexpression inhibited the endogenous telomerase activity. The overexpression of 3xFLAG-FL-hTERT, on the other hand, did not change the telomerase activity, which I tried to explain through an aberrant increase in endogenous β -containing hTERT transcripts following DOX-induction in FL12 cells. To delineate the differences in isoform-specific functions, a better-controlled method to overexpress hTERT isoforms should be devised. Importantly, I failed to observe any physical/functional association between the recombinant hTERT isoforms and mitochondria in hESCs. To validate this, future experiments should focus on investigating the phosphorylation status of hTERT and kinases involved in hTERT cross-membrane translocation. Additionally, differently tagged- (smaller tag size and C-terminal location), or non-tagged hTERT isoforms should be considered. Lastly, I demonstrated the pro-survival role of FL-hTERT and the pro-apoptotic role of β -hTERT in hESCs under H₂O₂-induced stress. Based on my results, previous literature, and the known/observed side effects of doxycycline, I conclude that more extensive assessment on intra-cellular ROS and ROS-induced damage is required for an appropriate interpretation of the results.

Despite the great amount of evidence pointing to the extra-telomeric roles of the general hTERT population, surprisingly little effort has been made to tease out the functional significance of different hTERT isoforms and the regulation of hTERT splicing. For better understanding of hTERT, more attention to developing better techniques and tools for the detection of all hTERT isoforms is required so that TERT isoform-specific functions can be elucidated.

Chapter 5: Reference List

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Appendix: Supplemental Figures and Tables

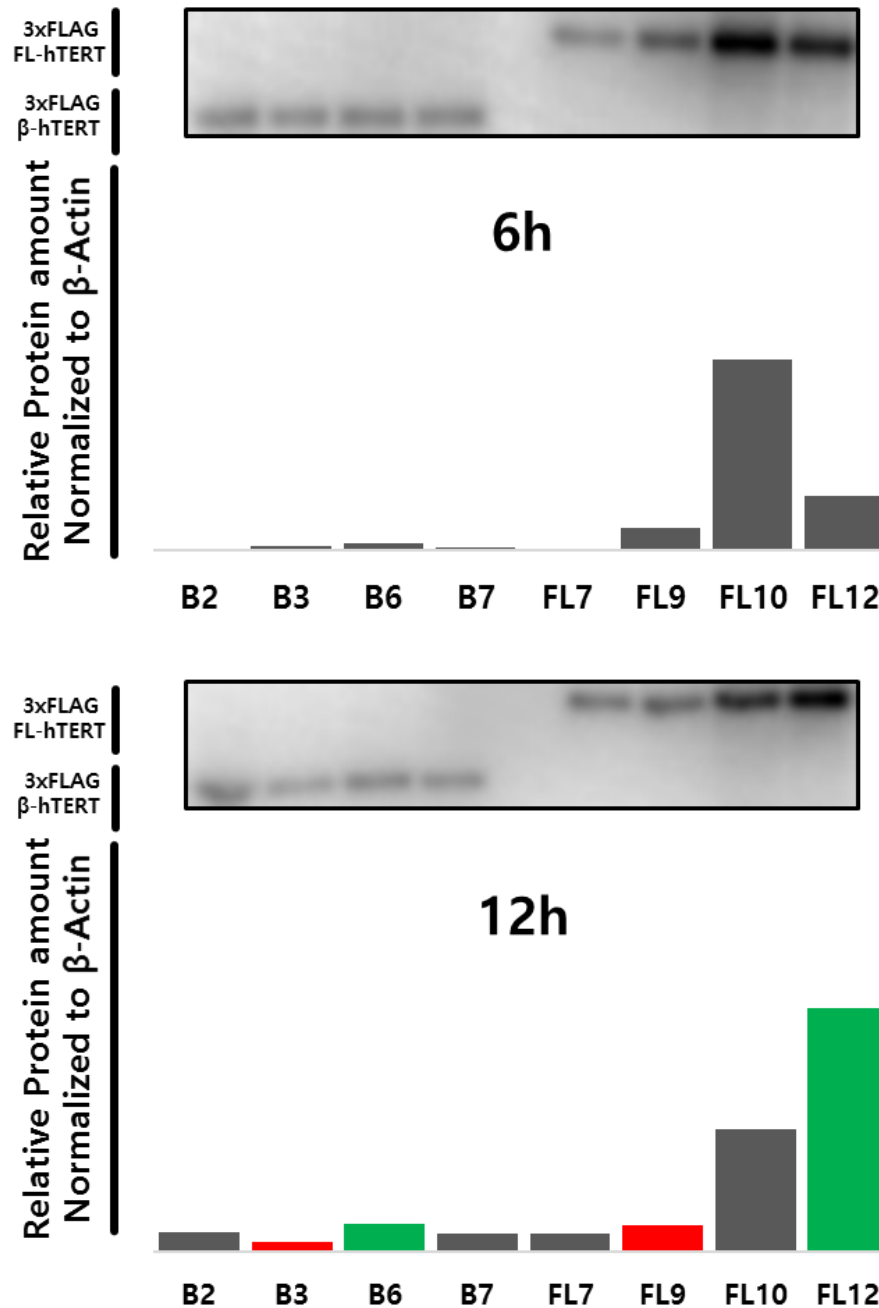
A1: Table 2.1 List of PCR primers

Primer Target	Sequence (Forward/Reverse)	Reference
Taqman $\alpha+\beta+$	TCAAGGTGGATGTGACGGG/ GGACTTGCCCCTGATGCG	[254]
Taqman $\beta-$	TCAAGGTGGATGTGACGGG/ GGCACTGGACGTAGGACGTG	[254]
Taqman $\alpha-$	CCTGAGCTGTACTTTGTCAAGGA/ GGACTTGCCCCTGATGCG	[254]
Taqman $\alpha-\beta-$	CCTGAGCTGTACTTTGTCAAGGA/ GGCACTGGACGTAGGACGTG	[254]
Taqman Probe	[6FAM] CGTGTCTCTGGGGTTTGATGATGCTGGCGA[6FAM]	[254]
β -GEO	CCTGCTGATGAAGCAGAACA/ TTGGCTTCATCCACCACATA	
TERC	CGCCTTCCACCGTTCATTCT/ GCTGACAGAGCCCAACTCTT	
RMRP	AAAGTCCGCCAAGAAGCGTA/ CTGCCTGCGTAACTAGAGGG	
β -actin	TCAAGATCATTGCTCCTCCTGAG/ ACATCTGCTGGAAGGTGGACA	
HPRT1	CCCTGGCGTCGTGATTAGTG/ TCGAGCAAGACGTTTCAGTCC	
TS	AATCCGTCGAGCAGAGTT	[191]
ACX	GCGCGGCTTACCCTTACCCTTACCCTAACC	[191]
TSNT	AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCG AT	[191]

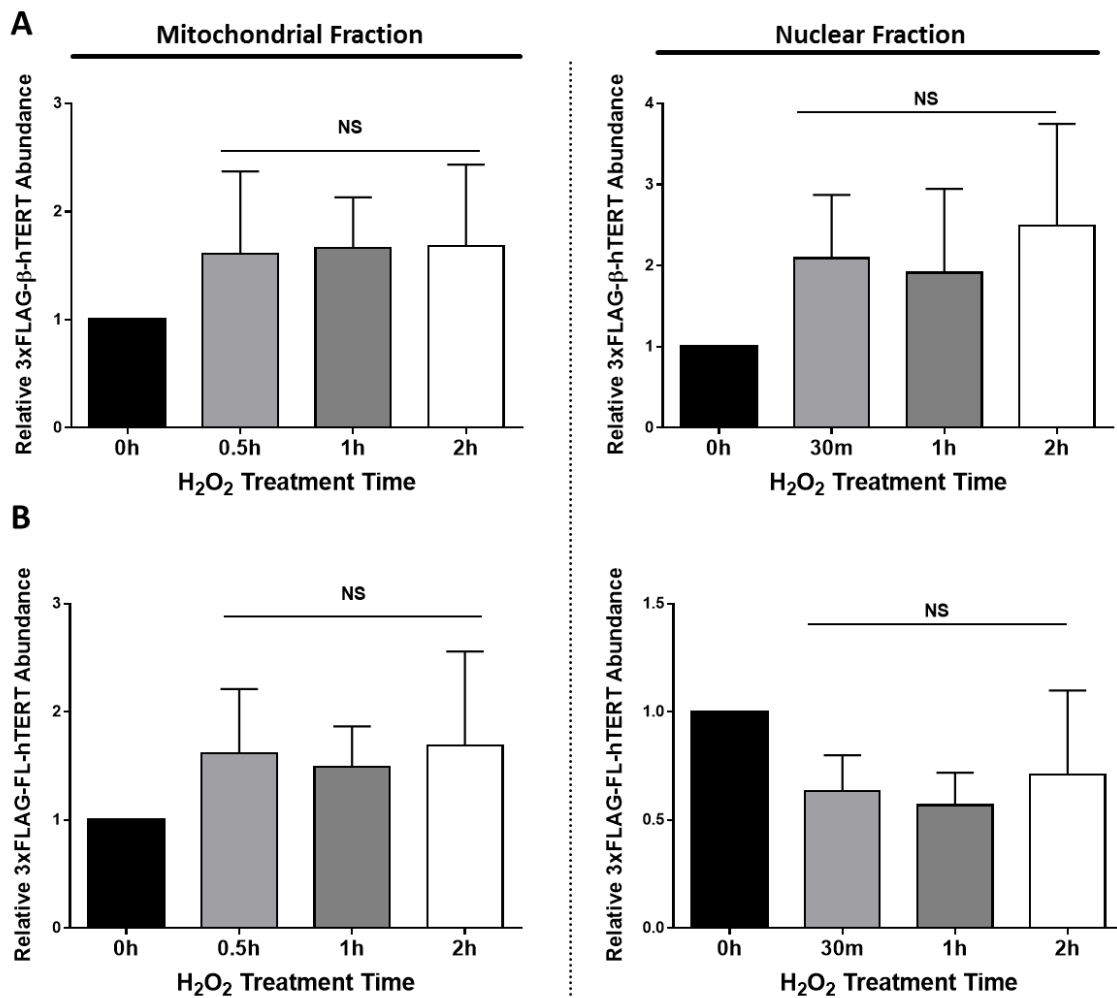
NT	ATCGCTTCTCGGCCTTTT	[191]
Mitochondrial tRNA	CACCCAAGAACAGGGTTTGT/ TGGCCATGGGTATGTTGTTA	[255]
B2M	CCAGCAGAGAATGGAAAGTCAA/ TCTCTCTCCATTCTTCAGTAAGTC	[255]
POLG	CATTGGACATCCAGATGCTG/ CCTGATATGAGCTCGGTCAA	
PGC1B	CCACATCCTACCCAACATCAAG/ CACAAGGCCGTTGACTTTTAGA	
NRF1	GGAGTGATGTCCGCACAGAA/ CGCTGTTAAGCGCCATAGTG	
TFAM	TATCAAGATGCTTATAGGGC/ ACTCCTCAGCACCATATTCT	
TF1BM	TCTGCAATGTTTCGACACATC/ ACCTATATAAGAAGCTCCAC	
SOD2	GGGATTGATGTGTGGGAGCA/ TGACTAAGCAACATCAAGAAATGC	
UCP2	ACAAGACCATTGCCCGAGAG/ AGGAGGGCATGAACCCTTTG	

A2: Table 2.2 List of antibodies and dyes

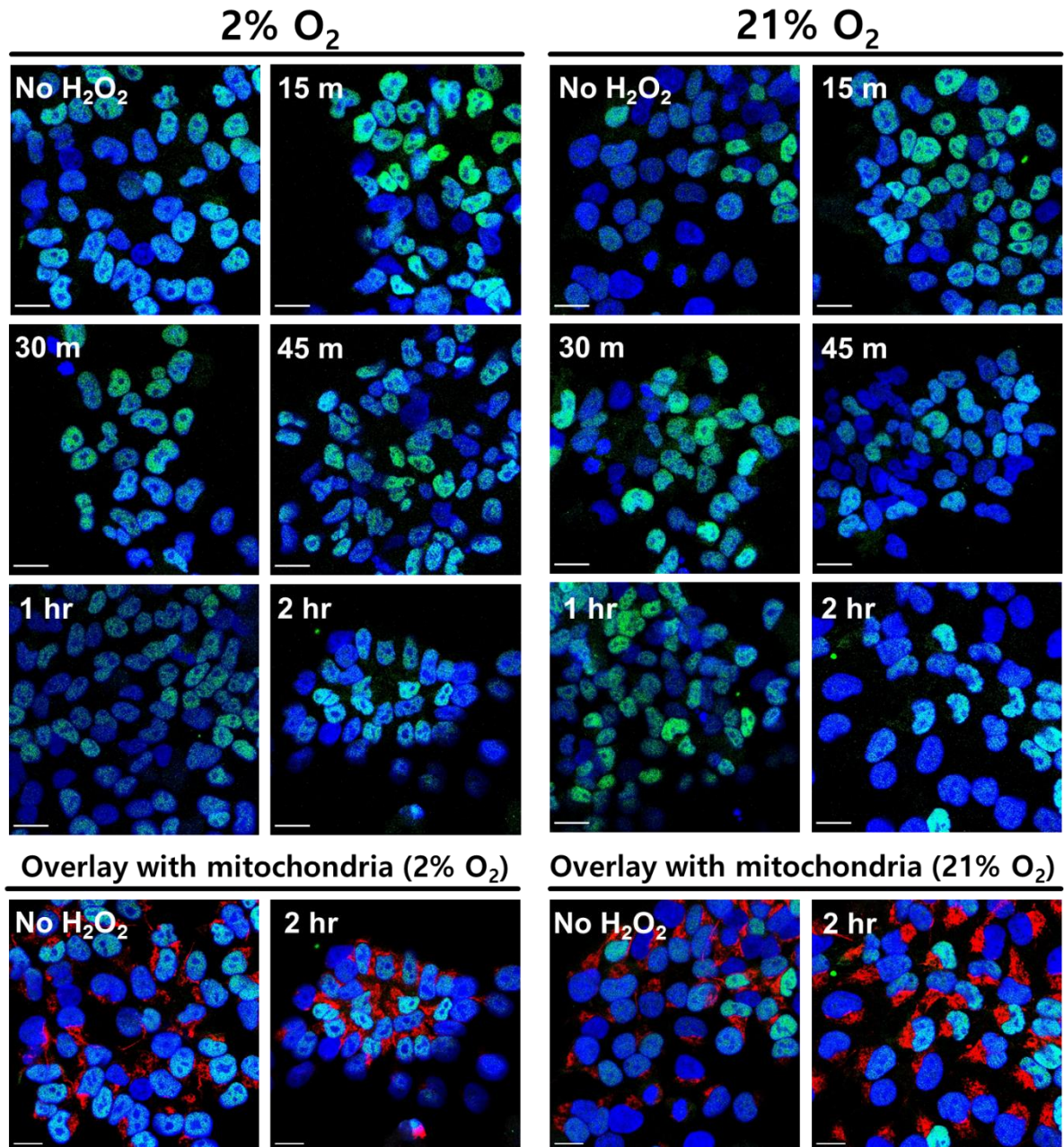
Target	Company	Host	Catalogue #
3xFLAG	Sigma	Mouse Monoclonal	F1084
β -ACTIN	Cell signaling	Rabbit Monoclonal	5125S
Histone H3	Cell signaling	Rabbit Monoclonal	4499
Prohibitin	Abcam	Mouse Monoclonal	ab55618
VDAC	New England Biolabs	Rabbit Monoclonal	4661S
TOM20	Protein Technologies	Rabbit Polyclonal	11802-1-AP
Alexa Fluor 488	Abcam	Goat	ab150113
Alexa Fluor 594	Abcam	Goat	ab150080
MitoTracker CMX ROS	ThermoFisher	-	M7512
DAPI	ThermoFisher	-	R37606
JC-1	ThermoFisher		T3168
Calcein AM	eBioscience	-	65-0853-39
7-AAD	eBioscience	-	00-6993
Annexin V-FITC	ThermoFisher	-	A13199



A3: Fig. 3.1 The amount of 3xFLAG-hTERT in the respective HES2 clones after 6-hours and 12- hours post doxycycline induction. 3xFLAG-hTERT isoform expression was induced via 1 μ g/ml doxycycline (6 and 12 hours) in HES2 transgenic clones grown under normoxia (21% O₂). Cells were harvested and processed for Western blot. Membranes were probed for 3xFLAG-hTERT isoforms using M2 anti-FLAG mouse monoclonal antibodies (Sigma). The protein level of 3xFLAG-hTERT isoforms in each clone at each time point of doxycycline treatment was compared by Western blot & densitometry analysis. Clonal names are in forms of B# for 3xFLAG-β-hTERT-expressing lines, and FL# for 3xFLAG-FL-hTERT-expressing lines.



A4: Fig. 3.2 H₂O₂-induced oxidative stress and its effect on the cellular localization of 3xFLAG-hTERT. Cells were grown under 2% O₂ and were treated with 1 μg/ml DOX for two days prior to harvest. 100 μM H₂O₂ was applied at different times (30m, 1hr, 2hr) prior to harvest. Mitochondria- and Nuclei-enriched fractions were isolated by differential centrifugation. The amount of **A**) 3xFLAG-β-hTERT and **B**) 3xFLAG-FL-hTERT in mitochondrial (left side)/nuclear (right side) fractions was investigated by Western blot (data not shown)/densitometry analysis. Results were normalized to prohibitin and histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to H₂O₂ negative control n=3, error bar=±SEM. NS = non-significant.



A5: Fig. 3.3: The effect oxygen tension on the intra-cellular localization of 3xFLAG-FL-hTERT in hESCs. Transgenic hESCs were grown under 21% O₂ and 2% O₂. HES2 FL12 were treated with 1 μg/ml DOX for two days prior to harvest. 100 μM H₂O₂ was applied at different time points (15min, 30min, 45min, 1hr, 2hr prior to fixation). Immunofluorescence of 3xFLAG-FL-hTERT (green fluorescence), nuclei (DAPI; blue fluorescence), and mitochondria (TOM20; red) was visualized with a confocal microscope (Zeiss LSM 5 Duo Vario). Images were taken using 63X oil-immersion objective lens and x10 eyepiece lens. Scale bars = 20 μm.

Education

- 2006 - 2012 B.Sc. (Honors) in Biochemistry at University of Guelph, Ontario, Canada
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- 2013 Lab internship at an embryology laboratory
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- Jan 2014 - M.Sc. student at University of Western Ontario,
Department of Physiology and Pharmacology.

Relevant Laboratory Skills

- Mammalian Cell/Micro-organism culturing
- Embryo culturing/manipulation
- Basic lab techniques for molecular analysis (e.g. PCR, Immunoprecipitation, etc.)

Publication

J. H. Teichroeb, J. Kim, and D. H. Betts (2016), "The Role of Telomeres and Telomerase Reverse Transcriptase Isoforms in Pluripotency Induction and Maintenance," *RNA Biol.*, vol. 6286, no. January, pp. 00–00,.

Research Experiences

May 2012 – Aug 2012 | Research Project as undergraduate student, department of molecular and cellular biology, University of Guelph (supervised by Dr. Jnanankur Bag)

Poly(A) binding proteins and the qualitative investigation of their interactions with translation regulators in HeLa.

Relevant techniques:

- Maintenance of cell culture (HeLa)
- Western Blot

Jul 2013 – Dec 2014 | Research Project at Theriogenology Lab, College of Veterinary Medicine, Gyeongsang National University, South Korea (supervised by Dr. Gyu-Jin Rho)

The effect of melatonin on proliferation/pluripotency of porcine mesenchymal stem cells (MSC):

Relevant techniques:

- Maintenance and directed differentiation of MSCs

Jan 2014 – Current | M.Sc. Student, Physiology and Pharmacology, University of Western Ontario, (supervised by Dr. Dean H. Betts)

FL- and β -variant of human telomerase reverse transcriptase in the regulation of mitochondria of human embryonic stem cell.

Relevant techniques:

- Maintenance of human embryonic stem cell
- Immunofluorescence (Confocal microscopy)
- Various types of PCR
- RNA-immunoprecipitation
- Cloning